

REMARKS/ARGUMENTS

Claims 23-24, 26, and 28-37 are pending in the above-identified application. Claims 25 and 27 have been withdrawn by the Examiner as being drawn to a non-elected invention. Claims 25 and 27 have been canceled without prejudice to Applicant's right to prosecute the subject matter of the claims in a related, co-pending application. Applicants respectfully request reconsideration of the pending claims in view of the below remarks.

Rejections Under 35 U.S.C. §103

Claims 23, 31-32, 33-37 remain rejected under 35 U.S.C. §103(a) as being obvious over Sullusto *et al.* (*J. Exp. Med.*, 179:1109-1118, 1994), in view of Bigotti *et al.*, (Prostate 19:73-87, 1991), as evidenced by Inaba *et al.*, (*J. Exp. Med.* 166:182-94, 1987), for reasons already of record in the office action dated June 2, 2006.

The Examiner has considered Applicants' last response but found it not to be persuasive. In particular, the Examiner alleges that contrary to Applicants argument, Bigotti *et al.* do not teach away from the claimed invention. The Examiner believes that although Bigotti *et al.* teach that there is no correlation between cytological tumor grade and the presence of Langerhans cells in close contact with the prostate cancer gland, Bigotti *et al.* do not teach that there is no correlation between tumor rejection and Langerhans cells.

Applicants must again disagree with the Examiner. Respectfully, Applicants fail to see where the artisan of ordinary skill would consider such an ambiguous teaching as merely finding Langerhans cell in some low grade prostate tumors as providing any reason to combine Bigotti *et al.* with any of the other cited references which are directed to the maturation of dendritic cell precursors. It is well known that Langerhans cells are a member of the dendritic cell family, but they are typically found in the epidermis. At the time of the present invention

Langerhans cells were thought to contact and process antigen in the epidermis and then migrate to draining lymph nodes where the Langerhans cells would contact naïve T cells. The presence of Langerhans cell in a sampling of prostate cancers suggests nothing regarding an antigen that the cells might be presenting, if any. It is just as likely that the Langerhans cells were merely passing through the prostate to a lymph node. There is no comparison with a sample of normal prostate to indicate whether the number of Langerhans cells is elevated from normal tissue. The artisan of ordinary skill has no reason to extend the teachings of Biogotti *et al.* beyond the use of the presence of Langerhans cells as a prognostic indicator.

Further, the Examiner believes that Bigotti *et al.* clearly teach that Langerhans cells are found mainly in low grade prostate cancer, as opposed to higher grades, and represent a good prognostic indicator (abstract, p.85). Still further, the Examiner asserts that Bigotti *et al.* teach that in this neoplastic environment, Langerhans cells act as antigen-presenting cells, while HLA II molecule may interact primarily or with the aid of Langerhans cells with macrophages and secondarily with T helper lymphocytes causing expansion of cytotoxic T cells, and enhancement of the antibody response to membrane-bound tumor associated antigens, therefore providing a means for controlling the escape of tumor cells from immune surveillance citing to p.85, paragraph under Conclusions. The Examiner has alleged that from the teaching of Bigotti *et al.*, one would have concluded that Langerhans cells act as antigen presenting cells, presenting prostate cancer antigens to the immune system, causing cytotoxic T cells expansion and enhancement antibody response to prostate cancer antigens, and thus play an important role in prostate cancer rejection, via eliciting an immune response. Still further, the Examiner asserts that although Bigotti *et al.* teach that this latter mechanism might be of secondary importance, in view of the paucity of lymphoid tissue within tumors (p.85, last two lines of the paragraph under Conclusions), this teaching actually provides motivation for one to make the prostate cancer antigen presenting cells *in vitro* to administer to a prostate cancer patient for augmenting or supplementing the numbers of available antigen presenting cells in the patient.

As above, the Examiner has merely asserted that the Langerhans cells found in the low grade prostate tumors of Bigotti *et al.* present a prostate antigen. The comments of the authors are merely speculative and have no factual basis that would provide the artisan of ordinary skill reason to draw the conclusions of the Examiner without the description provided by the specification as filed. Further, the quote from Bigotti *et al.* refers to membrane bound tumor associate antigens, not soluble antigen as required by the claims of the present invention. In addition, the quote cited by the Examiner does not refer to antigen specific cytotoxic T cells which typically would result from contact of an antigen presenting cell with a naïve T cell in a peripheral lymph node. Further, without additional information relating to, for example, the expression of B7-1 (CD80) and B7-2 (CD86), it is just as likely that the Langerhans cells are involved in a tumor escape mechanism. It was well known at the time of the invention that many tumors produced and induced the production of immunosuppressive cytokines, such as IL-10. Therefore, the presence of Langerhans cells, which can be characterized as immature dendritic cells would not be likely to successfully uptake and process antigen in the intratumoral environment. As such, the ordinary artisan would have no reasonable basis to combine the references as suggested by the Examiner.

The Examiner also argues that Applicant does not have any evidence that only cancer antigens directly on the cancer cells at the prostate cancer glands provide antigens for the antigen presenting cells. It is well known that immature dendritic cells, such as Langerhans cells, have the ability to capture antigen and process the antigen and efficiently present soluble antigen to specific T cells, as taught by Sallusto *et al.* In addition, the Examiner asserts that Applicants do not have any evidence that the tumor antigen exists only on the surface of cancer cells directly at the prostate cancer gland, and could not be also present at the peripheral border of the cancer gland, due to for example, the presence of antigens from cell membranes of lysed or necrotic cancer cells. Thus, the Examiner alleges that one cannot conclude from the mere absence of a correlation between cytological grade and the presence of Langerhans cells in contact with the prostate cancer gland that there is no correlation between tumor rejection and the presence of Langerhans cells in prostate cancer patients.

Applicants only commented as to the presence of cancer antigens directly on the cancer cells because the quote from Bigotti *et al.* cited by the Examiner states "HLA II molecule may interact primarily or with the aid of Langerhans cells with macrophages and secondarily with T helper lymphocytes causing expansion of cytotoxic T cells, and enhancement of the antibody response to membrane-bound tumor associated antigens, therefore providing a means for controlling the escape of tumor cells from immune surveillance" (emphasis added). It is this quote that suggested that the antigen would be membrane associated. As such, Applicants refer to the argument presented in the prior response regarding the such membrane associated antigens. Further, as above, it was well known that the intratumoral environment was immunosuppressive and could inhibit the ability of immature dendritic cells to uptake and process antigen. See for example the attached references, Steinbrink *et al.*, *Blood* 5:1634-1642, 1999, published subsequent to the earliest filing date of the present invention, and Pisa *et al.*, *Proc. Nat'l. Acad. Sci. USA* 89:7708-7712, 1992 and Smith *et al.*, *Amer. J. Pathol.* 145:18-25, 1994, cited therein. It would appear that the Examiner has used the specification as filed to construct a combination a references to try to support an obviousness rejection of the present invention using hindsight reconstruction.

In addition, the Examiner has asserted that although Bigotti *et al.* teach that macrophages play an important role in tumor rejection, Bigotti *et al.* do not exclude that Langerhans cells also play a role in tumor rejection and that one would have concluded from the teaching of Bigotti *et al.* and Sallusto *et al.* that Langerhans cells are capable of presenting antigens, including prostate cancer antigen, to immune cells, such as T cells, and eliciting an immune response, providing a means for controlling the escape of cancer cells from the immune surveillance, in view of that capturing and presenting antigen is a property of immature dendritic cells, such as Langerhans cells, in vitro and in vivo, as taught by Sallusto *et al.* The Examiner concluded that it would have been *prima facie* obvious to a person of ordinary skill in the art at the time the invention was made to obtain human, immature dendritic cells, using the method taught by Sallusto *et al.*, and to replace the antigen tetanus toxoid taught by Sallusto *et al.* with a prostate antigen taught by Bigotti *et al.*, for exposure of the prostate antigen to the immature

dendritic cells, because the dendritic cells, such as Langerhans' cells, would present prostate antigen to immune cells, and activate specific immune response, and thus, would provide treatment of prostate cancer. Further, the Examiner believes that one would have a reasonable expectation of success of making the claimed human dendritic cells, because the immature dendritic cells *in vitro*, obtained from culture in GM-CSF and interleukin-4, maintain the antigen capturing and processing capacity characteristics of immature dendritic cells *in vivo*, and efficiently present soluble antigen, as taught by Sallusto *et al.*

Again, Applicants must respectfully disagree with the conclusions of the Examiner. As above, the overall state of the art would not have provided sufficient reason for the artisan of ordinary skill to combine Bigotti *et al.* with Sallusto *et al.* with any reasonable expectation of success. Bigotti *et al.* merely provide evidence that the presence of Langerhans cells can be used as a possible prognostic indicator. As above, the overall state of the art would not provide the artisan of ordinary skill any reasonable basis for combining the references as suggested by the Examiner. In addition, Langerhans cells are considered immature dendritic cells and would not present antigen. It is well known in the art that immature dendritic cells process and only upon maturation present antigen to T cells. Further, as above, the intratumoral environment was known at the time of the present invention to secrete cytokines that are immunosuppressive. Still further, tetanus toxoid is a bacterial antigen, not a self antigen as is typical for a tumor associated antigen. Therefore, the skilled artisan had no reasonable basis to combine Bigotti *et al.* with Sallusto *et al.* Further, the references when considered alone do not render obvious the present invention.

As to the activation of CD4⁺ and CD8⁺ T cells, the Examiner has asserted that one would have concluded from the teaching of Bigotti *et al.* and Sallusto *et al.* that Langerhans cells are capable of presenting antigens, including prostate cancer antigen, to immune cells, such as T cells, and eliciting an immune response, providing a means for controlling the escape of cancer cells from the immune surveillance, *supra*. The Examiner has further noted that the dendritic cells taught by Sallusto *et al.*, and Bigotti *et al.*, (the Examiner has noted that Stites was not

intended to be included in the rejections under § 103) would activate CD4⁺ and/or CD8⁺ T cells, because activation of CD4⁺ and/or CD8⁺ T cells is a property of dendritic cells, as evidenced by Inaba *et al.* The Examiner further asserts that although the references do not specifically teach that the dendritic cells activate CD4⁺ and/or CD8⁺ T cells, however, the claimed dendritic cells appear to be the same as the dendritic cells taught by the combined art, absent a showing of unobvious differences.

Applicants again must respectfully disagree with the reasoning and conclusions of the Examiner. As above, Langerhans cells are considered immature dendritic cells and would not be capable of presenting any antigen, much less a prostate antigen. Further, although Inaba *et al.* may or may not teach that activation of CD4⁺ and/or CD8⁺ T cells is a property of dendritic cells, the artisan of ordinary skill has not to combine Bigotti *et al.* and therefore there is no disclosure in the cited references of the dendritic cells of the present invention. As such, the dendritic cells of the present invention and use of these cells in the activation of CD4⁺ and CD8⁺ T cells are not obvious.

Concerning the expectation that the human peripheral blood taught by Sallusto *et al.* is from normal donor individual, the Examiner summarizes Applicants response as asserting that the combination of Sallusto *et al.* and Bigotti *et al.* does not suggest replacing the tetanus toxoid antigen with any other antigen. Applicants' response is also alleged to assert that therefore, there is no teaching or suggestion by Sallusto *et al.* and Bigotti *et al.* of the use of normal or diseased peripheral blood as a source of dendritic cells. The Examiner alleges that the combination of Sallusto *et al.*, Bigotti *et al.* suggests the composition of the claimed invention, and that although Sallusto *et al.* do not explicitly state that the human peripheral blood is from a normal individual, Sallusto *et al.* do not state that the human peripheral blood is from a diseased individual. In addition, the Examiner alleges that the human peripheral blood taught by Sallusto *et al.* would be expected to be from a normal donor individual. In the alternative, the Examiner alleges that it would have been obvious to use the peripheral blood from normal healthy donor as

a source of immature dendritic cells, to increase the availability of the source of immature dendritic cells, in view of the teaching of Sallusto *et al.* and Bigotti *et al.*

Applicants again respectfully disagree with the reasoning of the Examiner. As above, there is no reason for the artisan of ordinary skill to combination the teachings of Bigotti *et al.* with those of Sallusto *et al.* The state of the art at the time of the present invention does not provide the artisan with any reason to look to the teaching of Sallusto *et al.* when looking for Langerhans cells in a prostate tumor sample to diagnose or stage a tumor sample. Nor is there any reason for the artisan to look to Bigotti *et al.* for guidance when reviewing the teachings of Sallusto *et al.* Whether immature dendritic cells isolated from normal or diseased individuals is not relevant as there is no reasonable basis to combine Bigotti *et al.* to replace tetanus toxoid with a prostate antigen as suggested by the Examiner as set forth above.

Claim 24 remains rejected under 35 U.S.C. §103(a) as being obvious over Sallusto *et al.*, in view of Bigotti *et al.*, as evidenced by Inaba *et al.*, *supra*, and further in view of Cohen *et al.*, (*Cancer Res.* 54:1055-1058, 1994). The teachings of Sallusto *et al.*, Bigotti *et al.*, and Inaba *et al.* have been set forth above. Further, the Examiner has acknowledged that Sallusto *et al.*, Bigotti *et al.*, and Inaba *et al.* do not teach that the antigen is a lysate of prostate tumor cells. The Examiner believes that Cohen *et al.* teach that syngenic dendritic cells, when pulsed with tumor lysate, induce antigen-specific proliferation of antitumor CD4⁺ T cells, relevant to the rejection of the syngenic methylcholanthrene tumor (abstract) and that it would have been obvious to use as prostate antigen, a lysate of prostate cancer cells from a prostate cancer patient, because prostate cancer cells would have several prostate cancer-specific antigens. The Examiner also believes that it would have been obvious to use a tumor lysate because Cohen *et al.* teach that a tumor lysate successfully primes the dendritic cells for inducing antigen-specific proliferation of antitumor CD4⁺ T cells and the Examiner believes that it would be more convenient to use tumor lysate because it does not require the extra step of purification of the antigen.

As above, the combination of Sallusto *et al.* and Bigotti *et al.* fail to teach the compositions of the present invention. Instead, Bigotti *et al.* teach that macrophage likely induce the immune response seen in prostate cancer. As such, any combination with Inaba *et al.* and/or Cohen *et al.* can not provide the skilled artisan with incentive to combine the references to use a lysate of prostate cancer cells from a prostate cancer patient to make the compositions of the claim 24.

Claim 26 remains rejected under 35 U.S.C. §103(a) as being obvious over Sallusto *et al.*, in view of Bigotti *et al.*, and as evidenced by Inaba *et al.*, *supra*, as applied to claim 23, and further in view of Lutz *et al.* (of record). The teachings of Sallusto *et al.*, Bigotti *et al.*, and Inaba *et al.* as summarized by the Examiner have been set forth above. Although the Examiner has concluded that Sallusto *et al.*, Bigotti *et al.* and Inaba *et al.* do not teach dendritic cells that are extended life span dendritic cells, the Examiner alleges that Lutz *et al.* teach making immortalized dendritic cells (Abstract), which overcomes the problem of being unable to maintain dendritic cells *in vitro* for long periods of time (p. 278). Therefore, the Examiner believes that it would have been *prima facie* obvious to a person of ordinary skill in the art at the time the invention was made to immortalize the dendritic cells taught by Sallusto *et al.*, Bigotti *et al.*, and Inaba *et al.*, using the immortalizing method taught by Lutz *et al.*, because immortalizing dendritic cells would enable maintenance of dendritic cells *in vitro* for long periods of time, as taught by Lutz *et al.*

As above, the Sallusto *et al.*, Bigotti *et al.* and/or Inaba *et al.* when considered either alone or in combination do not teach the compositions of the present invention. As such, the addition of Lutz *et al.* allegedly teaching immortalization of dendritic cells can not provide the skilled artisan with motivation to, or reasonable expectations of excess to, make the composition as set forth in claim 26.

Claims 28-29 remain rejected under 35 U.S.C. §103 as being obvious over Sallusto *et al.*, Bigotti *et al.*, Inaba *et al.*, and Cohen *et al.*, *supra*, as applied to claim 23, and

further in view of Taylor *et al.* (of record). The teachings of Sallusto *et al.*, Bigotti *et al.*, Inaba *et al.* and Cohen *et al.* as set forth by the Examiner have been set forth above. Although the Examiner acknowledges that Sallusto *et al.*, Bigotti *et al.*, Inaba *et al.*, and Cohen *et al.* do not teach that the dendritic cells are cryopreserved, the Examiner believes that Taylor *et al.* teach cryopreservation of dendritic cells, wherein the cryopreserved dendritic cells can be used in immunological procedures. As such, the Examiner believes that it would have been *prima facie* obvious to a person of ordinary skill in the art at the time the invention was made to cryopreserve the dendritic cells taught by Sallusto *et al.*, Bigotti *et al.*, Stites (was Inaba *et al.* intended?), and Cohen *et al.*, using the cryopreservation method taught by Taylor *et al.*, to preserve the previously isolated dendritic cells for later use.

As above, Sallusto *et al.*, Bigotti *et al.* and Inaba *et al.*, do not teach the compositions of the present invention. Taylor *et al.* is directed to cryopreservation techniques and does not address the teachings of Bigotti *et al.* Bigotti *et al.* teaches that the presence of Langerhans cells in prostate tumor can be of diagnostic significance and speculates that an immune response might be induced in prostate cancer by macrophage. As such, Sallusto *et al.*, Bigotti *et al.* and Inaba *et al.*, when considered individually or in any combination do not teach or suggest the compositions as set forth in claims 28 and 29.

Claim 30 remains rejected under 35 U.S.C. §103 as being obvious over Sallusto *et al.*, Bigotti *et al.*, and Inaba *et al.*, *supra*, as applied to claim 23, and further in view of Taylor *et al.* (of record), as applied to claim 28, and Lutz *et al.*, (of record). The teachings of Sallusto *et al.*, Bigotti *et al.*, Inaba *et al.* and Taylor *et al.* as set forth by the Examiner have been set forth above. The Examiner has noted that Sallusto *et al.*, Bigotti *et al.*, Inaba *et al.* and Taylor *et al.* do not teach that the dendritic cells have extended life, but the Examiner alleges that Lutz *et al.* teach making immortalized dendritic cells (Abstract), which overcomes the problem of being unable to maintain dendritic cells *in vitro* for long periods of time (p. 278). As such, the Examiner believes that it would have been *prima facie* obvious to a person of ordinary skill in the art at the time the invention was made to immortalize the cryopreserved dendritic cells taught

by Sallusto *et al.*, Bigotti *et al.*, Inaba *et al.* and Taylor *et al.*, using the immortalizing method taught by Lutz *et al.*, because immortalizing dendritic cells would allow maintenance of dendritic cells *in vitro* for long periods of time, as taught by Lutz *et al.*

As above, the teachings of Sallusto *et al.*, Bigotti *et al.*, Inaba *et al.* and Taylor *et al.* do not disclose or suggest the compositions of the present application. The teachings of Lutz *et al.* when considered either alone or in combination with any of the other cited references does not cure the deficiencies of the primary references, Sallusto *et al.* and Bigotti *et al.* in that Bigotti *et al.* when viewed in light of the status of the art as a whole do not present any reasonable evidence that any Langerhans cells found in prostate were presenting any antigen, much less a prostate antigen, and therefore the artisan or ordinary skill would have no reason to combine the teachings of Bigotti *et al.* with those of Sallusto *et al.* or any of the other cited references to obtain the compositions of the present invention.

In view of the above remarks, Applicants respectfully request the Examiner to reconsider and withdraw the various rejections of claims 23, 24, 26, and 28-37 under 35 USC § 103(a) as being obvious over Sallusto *et al.*, Bigotti *et al.* as evidenced by Inaba *et al.*, in view of Stites, and Cohen *et al.*, alone and in various combinations. In particular, Bigotti *et al.* teaches away from the compositions of the present invention by teaching that the immune response to prostate cancer is likely induced by macrophage. In light of the teachings of Bigotti *et al.* the skilled artisan would not have been motivated to produce the compositions of the present invention.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested.

GERALD P. MURPHY *et al.*
Application No. 09/016,737
Amendment under 37 CFR 1.116 Expedited Procedure
Examining Group 1642

PATENT

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,

Dated: 15 August 2007

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ATTACHMENT 1

Steinbrink, et al.

Interleukin-10-Treated Human Dendritic Cells Induce a Melanoma-Antigen-Specific Anergy in CD8⁺ T Cells Resulting in a Failure to Lyse Tumor Cells

By Kerstin Steinbrink, Helmut Jonuleit, Gabriele Müller, Gerold Schuler, Jürgen Knop, and Alexander H. Enk

Dendritic cells (DC) are critically involved in the initiation of primary immune processes, including tumor rejection. In our study, we investigated the effect of interleukin-10 (IL-10)-treated human DC on the properties of CD8⁺ T cells that are known to be essential for the destruction of tumor cells. We show that IL-10-pretreatment of DC not only reduces their allostimulatory capacity, but also induces a state of alloantigen-specific anergy in both primed and naive (CD45RA⁺) CD8⁺ T cells. To investigate the influence of IL-10-treated DC on melanoma-associated antigen-specific T cells, we generated a tyrosinase-specific CD8⁺ T-cell line by several rounds of stimulation with the specific antigen. After coculture with IL-10-treated DC, restimulation of the T-cell line with un-

treated, antigen-pulsed DC demonstrated peptide-specific anergy in the tyrosinase-specific T cells. Addition of IL-2 to the anergic T cells reversed the state of both alloantigen- or peptide-specific anergy. In contrast to optimally stimulated CD8⁺ T cells, anergic tyrosinase-specific CD8⁺ T cells, after coculture with peptide-pulsed IL-10-treated DC, failed to lyse an HLA-A2-positive and tyrosinase-expressing melanoma cell line. Thus, our data demonstrate that IL-10-treated DC induce an antigen-specific anergy in cytotoxic CD8⁺ T cells, a process that might be a mechanism of tumors to inhibit immune surveillance by converting DC into tolerogenic antigen-presenting cells.

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DENDRITIC CELLS (DC) are highly specialized antigen-presenting cells (APC) of the immune system.¹ Their strategic positioning in nonlymphoid tissue and their ability to circulate via blood and lymph to lymphoid organs after antigen stimulation demonstrate their important role in the induction of immune responses against invading pathogens.^{2,3} During their migration, DC such as Langerhans cells (LC) are thought to undergo characteristic modulations of function and phenotype.^{4,5} Locally produced inflammatory cytokines and the encounter with an antigen promote the maturation and migration of DC to regional lymph nodes. During this process DC undergo a differentiation from a processing to a presenting functional cell type, characterized by the expression of costimulatory molecules, cytokine production, and a typical morphology.⁵⁻⁷ In contrast to other types of APC, fully mature DC are potent activators of naive T cells and are regarded as important initiators of primary specific immune responses.¹

Tumors use various strategies for escape from immunologic recognition or destruction.⁸ One of the potential escape mechanism is the production of the immunosuppressive cytokine interleukin-10 (IL-10) by the tumor cells themselves or the induction of such factors in tumor-infiltrating cells. The release of IL-10 from many tumors, such as human renal, colon, ovarian, lung, and basal cell carcinomas, brain neoplasms, and Epstein-Barr virus-transformed B lymphomas, has been reported.⁹⁻¹⁴ Furthermore, in malignant melanoma cells, the

expression of IL-10 mRNA or protein was demonstrated.^{10,15-17} Studies in progressing tumors showed a correlation of an advanced course of the disease with elevated IL-10 serum levels, a preferential detection of IL-10 secreting tumor cells in metastatic lesions, and a higher release of IL-10 by metastatic cells from patients with progressive melanoma metastases compared with metastases responding to therapy.¹⁸⁻²⁰

The immunosuppressive properties of IL-10 have been well documented in several studies. An inhibitory effect on the function of APC and T cells has been described. The inhibitory influence of IL-10 on the APC function of DC and macrophages is due to the downregulation of major histocompatibility complex (MHC) class II and several costimulatory molecules and the reduction of a variety of secreted inflammatory cytokines.²¹⁻²⁸ With regard to tumor rejection, it was demonstrated that IL-10 inhibits tumor antigen presentation by epidermal APC in the murine system.²⁹ More importantly, it was shown that IL-10-treated LC induce an antigen-specific tolerance in Th1 cells, but not in Th2 cell clones.³⁰

Recently, we demonstrated that IL-10-treated human DC, generated from peripheral blood, induce a state of antigen-specific anergy in various populations of CD4⁺ T cells.³¹

In the present study, we investigated the effect of IL-10-treated DC on the function of CD8⁺ T cells to evaluate a potential mechanism of tumor cells to inhibit the cytotoxic capacity of T cells by anergy induction. We demonstrate that IL-10-treated DC show a reduced capacity to stimulate the proliferation of both primed and naive CD8⁺ T cells in allogeneic MLR and anti-CD3 assays. More interestingly, IL-10-treated DC induce a state of alloantigen-specific anergy in CD8⁺ T cells. To investigate the influence on melanoma-associated antigen-specific T cells, the effect of IL-10-treated DC on a tyrosinase-specific T-cell line was observed. Restimulation of the specific T cells with untreated, antigen-pulsed DC demonstrated a peptide-specific anergy in the tyrosinase-specific CD8⁺ T cells. In contrast to optimally stimulated CD8⁺ T cells, tyrosinase-specific anergic CD8⁺ T cells failed to lyse an HLA-A2⁺, tyrosinase-expressing melanoma cell line. In conclusion, production of IL-10 by tumor cells may reflect an escape mechanism from immune surveillance by converting DC into tolerogenic APC.

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MATERIALS AND METHODS

Preparation of DC. Blood-derived DC were prepared according to a modified protocol originally described by Romani et al.³² Briefly, whole blood was heparinized and separated by a Ficoll gradient. Peripheral blood mononuclear cell fractions were then depleted of T and B cells using immunomagnetic beads coated with anti-CD2 and anti-CD19 monoclonal antibody (MoAb; Dynal, Oslo, Norway). The remaining cells were cultured in X-VIVO 15 (Biowhittaker, Walkersville, MD) in 6-well-plates (Costar, Cambridge, MA) for 7 days. Cultures were supplemented with 1,000 U/mL human IL-4 (hIL-4; PBH, Hannover, Germany), 800 U/mL human granulocyte-macrophage colony-stimulating factor (hGM-CSF; Leukomax; Sandoz, Basel, Switzerland), and 1% autologous plasma. Cells were fed with fresh medium every 2 days. On day 7, nonadherent cells were rinsed off the plates and resuspended in fresh complete medium with GM-CSF and IL-4 and additionally stimulated with IL-1 β (10 ng/mL), tumor necrosis factor- α (TNF- α , 10 ng/mL; PBH), IL-6 (1,000 U/mL; R&D Systems, Wiesbaden, Germany), and prostaglandin E₂ (PGE₂; 1 μ g/mL; Sigma, München, Germany) to induce and stabilize the maturation of DC. DC were harvested for fluorescence-activated cell sorting (FACS) analysis or functional tests 3 to 5 days after resuspension and stimulation. Simultaneously to the addition of the stimulating mixture of cytokines and PGE₂, IL-10 (40 ng/mL; DNAX, Palo Alto, CA) was added to the culture for the last 2 days of culture. For the study of kinetics, IL-10 was added at various time points (as indicated).

T-cell purification, allogeneic proliferation, and anti-CD3 assay. T cells were prepared from human blood using Ficoll gradients and subsequent purification by antibody-coated immunomagnetic beads (MACS Systems; Miltenyl, Bergisch Gladbach, Germany) according to standard protocols (purity of >95% CD8⁺ T cells and >90% CD45RA⁺ T cells). In some experiments, cord blood was used as the source of naive CD8⁺ T cells. Purity was tested using FACS analysis.

DC were prepared as described above and cocultured with 2×10^4 T cells per well in 96-well plates (Costar). For anti-CD3 assays anti-CD3 MoAb (OKT3, ATCC, CRL 8001) was used at a titrated dilution of a hybridoma supernatant (1:20). After 2 days (anti-CD3 assay) or 4 days (allogeneic MLR), the cells were pulsed with 1 μ Ci of [³H] TdR ([methyl-³H]thymidine) for 16 hours, harvested, and counted. Tests were performed in triplicate, and results were expressed as the mean cpm \pm standard deviation (SD).

Tyrosinase-specific T-cell lines (CTL). Naive CD8⁺ T cells (2×10^5) from HLA-A2* donors (purity of >95% CD8⁺ T cells, generated as described above) were cultured in X-vivo 20 (Biowhittaker) and stimulated with mature, HLA-A2*, autologous DC (2×10^4) pulsed with the specific tyrosinase peptide (YMDGTMSQV; 20 μ g/mL). After several restimulations (5 to 8 times) every 7 days and expansion of the cell number by addition of IL-2 (10 U/mL), the peptide-specific proliferation was tested using specific (tyrosinase) and unspecific (MART-1 [EAAGIGLTV], MAGE-1 [EADPTGHSY]) melanoma-associated antigens. Before their use in experiments, T cells were rested 7 to 8 days after the last addition of antigen and stimulator cells. Three tyrosinase-specific CD8⁺ T-cell lines from 2 unrelated HLA-A2* donors were generated and used for the experiments. MART-1-specific T-cell lines served as controls.

Anergy assay. Allogeneic CD8⁺ T cells or tyrosinase-specific CD8⁺ T cells were prepared as described above. T cells were cocultured during the first incubation at a density of 2×10^4 (allogeneic CD8⁺) or 2×10^4 (tyrosinase-specific CD8⁺ T cells) with 1×10^4 or 1×10^3 DC, pretreated with IL-10 (40 ng/mL), or untreated. In some experiments, anti-CD3 MoAb was added at a titrated dilution of a supernatant (as described above). Thirty-six hours later, T cells were separated by Histopaque and rested for 1 to 7 days in culture medium containing 2 U/mL IL-2. Subsequently, T cells were restimulated with DC generated from the same donor as used for the first culture in experiments with

CD8⁺ T cells or with DC generated from an HLA-A2* donor. Proliferation was measured 48 hours later by thymidine incorporation. Tests were carried out in triplicates, and results were expressed as mean cpm \pm SD. Additionally, cytokine production was measured by enzyme-linked immunosorbent assay (ELISA) in supernatants of restimulated cultures 48 hours after the beginning of culture.

Cytotoxicity assay. Cytotoxic activity was measured in a standard 4-hour assay using the ⁵¹Cr-labeled tyrosinase-expressing and HLA-A2* melanoma cell line SK-MEL 28 (provided by Dr T. Wölfel, Mainz, Germany) as targets. Briefly, 3×10^5 ⁵¹Cr-labeled tumor cells were cultured with tyrosinase-specific, HLA-A2* CD8⁺ CTL, precultured with mature or IL-10-treated DC, in effector:target ratios as indicated for 4 hours at 37°C. Tyrosinase-specific CD8⁺ T cells cocultured with the unrelated peptide MART-1 and untreated or IL-10-treated DC or HLA-mismatched DC (HLA-A1*) during the primary culture were used as controls.

Additional control experiments were performed using DC (HLA-A2*) as target cells pulsed with various peptides: tyrosinase (HLA-A2-restricted presentation), MART-1 (HLA-A2-restricted presentation), or MAGE-1 (HLA-A1-restricted presentation). Similar to the experimental setting described above, these ⁵¹Cr-labeled target cells were cocultured with the tyrosinase-specific, HLA-A2* CD8⁺ CTL, precultured with mature or IL-10-treated DC, for 4 hours at 37°C.

The percentage of specific lysis was calculated from the average of triplicates as $100 \times \frac{(\text{⁵¹Cr-release into supernatant} - \text{spontaneous release})}{(\text{total release in detergent} - \text{spontaneous release})}$. All synthetic peptides were tested for nonspecific lysis of target cells in the absence of cytotoxic T lymphocytes.

Cytokine analysis. For assessment of cytokine production, supernatants were collected 48 hours after restimulation of allogeneic-specific/tyrosinase-specific CD8⁺ T cells with mature, untreated DC and stored at -70°C. Amounts of IL-2, IL-4, IL-10, transforming growth factor- β (TGF- β), and interferon- γ (IFN- γ) were measured by ELISA using commercially available antibodies and standards according to the manufacturer's protocols (Pharmingen, Hamburg, Germany).

RESULTS

Inhibition of the allogenein-induced or anti-CD3-induced proliferation of CD8⁺ T cells after coculture with IL-10-treated DC. To evaluate the effect of IL-10 on the stimulatory capacity of DC, precursors of DC, generated from peripheral blood, were cultured for 7 days as described above and subsequently stimulated for the last 2 days with the defined cytokine cocktail (IL-1 β , TNF- α , and IL-6) and PGE₂ alone or additionally treated with IL-10 (40 ng/mL) and used as APC in variable numbers in allogenein-induced or anti-CD3-induced proliferation assays (Fig 1A and B). Human allogeneic CD8⁺ T cells, purified from peripheral blood or in some experiments from cord blood, were used as responder cells. A significant inhibition of the proliferation was demonstrated in all T-cell:DC ratios, if IL-10-treated DC were set in as APC both in allogenein-induced and in anti-CD3-induced proliferation assays. The reduced proliferation was demonstrated, if naive CD8⁺/CD45RA⁺ T cells (Fig 1A and B) or activated CD8⁺/CD45RO⁺ T cells (data not shown) were cocultured with IL-10-treated DC.

The effect of IL-10 on the DC was inhibited by the simultaneous addition of an anti-IL-10 antibody (10 μ g/mL; R&D Systems, Wiesbaden, Germany) to the culture (data not shown).

Mature DC are resistant to the effect of IL-10. Prior experiments had shown that fully mature DC were generated

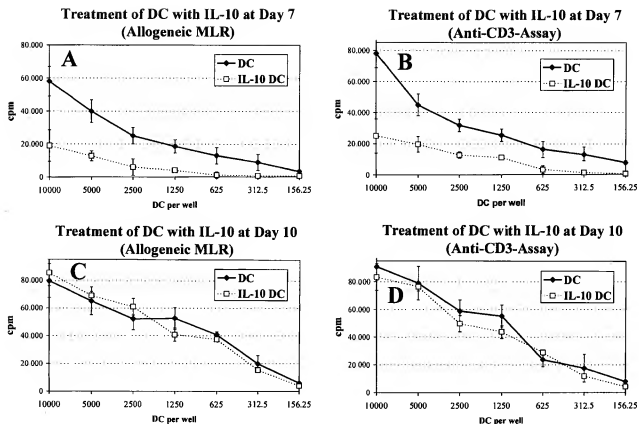


Fig 1. Inhibitory effects of IL-10-pretreated DC on alloantigen- or anti-CD3-induced proliferation of naive CD8⁺ T cells: dependence on the state of maturation of DC. DC were generated from peripheral progenitors as described above, resuspended at day 7, and stimulated with IL-1 β , IL-6, TNF- α , and PGE₂ to induce and stabilize the maturation of the DC. IL-10 was added for the last 2 days of culture at various time points after the stimulation: simultaneous addition at day 7 and harvested of the DC at day 9 (A and B) or addition at a later stage of maturation (day 10) and subsequently harvest at day 12 (C and D). Control and IL-10-treated DC were cocultured with naive CD45 RA⁺/CD8⁺ T cells (2×10^5) in allogeneic MLR (A and C) or anti-CD3 assays (B and D). Proliferation was measured by [³H] TdR uptake. The results are representative of five experiments.

after 11 to 14 days of culture after stimulation with IL-1 β , IL-6, TNF- α , and PGE₂ at day 7 and that these cells induced the most effective proliferative T-cell response.³³ In our experiments, IL-10 was added at various time points after the stimulation of the DC to determine whether IL-10 would affect the function of DC at all stages of their differentiation. The addition of IL-10 was most effective if DC at days 7 to 9 of culture were used, whereas fully mature DC at days 10 to 13 of culture were completely resistant to the effect of IL-10 (Fig 1). As an example, no inhibition of proliferation was observed if DC at day 12 of culture, after treatment with IL-10 at day 10, were cocultured with CD8⁺ T cells both in alloantigen-induced or anti-CD3-induced proliferation assays (Fig 1C and D).

Induction of alloantigen-specific anergy in CD8⁺ T cells by IL-10-treated DC. To test whether IL-10-treated DC would induce a state of alloantigen-specific anergy in CD8⁺ T cells, we performed a two-step anergy assay. In these experiments, alloantigen CD8⁺ T cells were cocultured with untreated or IL-10-treated DC. After the first coculture, T cells were rescued, cultured for 36 hours in the presence of IL-2 (10 U/mL), and subsequently restimulated with untreated, fully mature DC.

CD8⁺ T cells, cultured with untreated DC during the first

coculture, showed a vigorous proliferation to restimulation with mature DC during the second coculture (Fig 2). In contrast, CD8⁺ T cells cocultured with IL-10-treated DC were unresponsive to further stimulation with APC, if these cells were generated from the same donor as used in the first coculture (Fig 2). To determine whether this induction of anergy was antigen-specific, DC from a second, unrelated donor were used for restimulation. In these experiments, an unrestricted proliferation of the CD8⁺ T cells was observed independently of a pretreatment with IL-10 (Fig 2).

All experimental groups of T cells responded vigorously to IL-2, a cytokine that has been described to overcome the state of anergy (Fig 2).³⁰

To assess the kinetics of the induction of this T-cell anergy, CD8⁺ T cells rescued after the first coculture were cultured for various time periods (up to 7 days) in the presence of IL-2 (10 U/mL) before restimulation with untreated mature DC. In all experiments performed, the T cells showed a markedly reduced proliferation if IL-10-treated DC were used in the primary culture and the APC for both cultures were derived from the same donor (data not shown).

These experiments demonstrate that IL-10 converts the

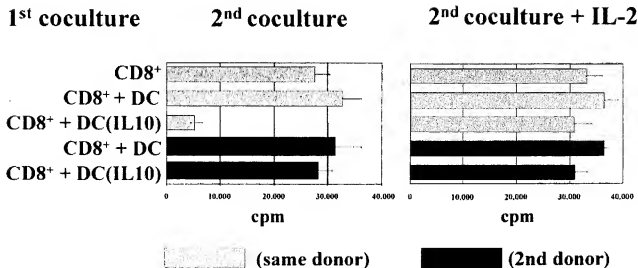


Fig 2. Induction of alloantigen-specific anergy in CD8⁺ T cells by IL-10-treated DC. Purified naive CD8⁺ T cells were cultured in medium alone; with untreated, control DC; or with DC pretreated with IL-10 (first coculture). After 36 hours, T cells were rescued, cultured for 1 to 7 days in medium containing low levels of IL-2 (2 U/mL), and subsequently restimulated (second coculture) with untreated, mature DC generated from the same donor (□) or a second unrelated donor (■). [³H] TdR incorporation was determined after 48 hours. The left bars show the antigen-specific response and the right bars show the T-cell response to IL-2 (100 U/mL).

function of DC to anergy-inducing cells and that this state of T-cell anergy is alloantigen-specific.

Inhibition of peptide-specific proliferation of a tyrosinase-specific cytotoxic CD8⁺ T-cell line. Cytotoxic CD8⁺ T cells are important for the lysis and elimination of tumors cells. To analyze the effect of IL-10-treated DC on the function of cytotoxic, melanoma-associated antigen-specific CD8⁺ T cells, we established three tyrosinase-peptide-specific CD8⁺ T-cell lines from two unrelated HLA-A2⁺ donors. Cytotoxic CD8⁺ T cells were used after 5 to 8 weekly restimulations with

peptide-pulsed autologous DC in the presence of low levels of IL-2 (10 U/mL).

We assessed the proliferation of the tyrosinase-specific CD8⁺ T-cell line after coculture with untreated versus IL-10-treated tyrosinase peptide-pulsed DC (Fig 3). Strong peptide-specific proliferation was observed in cultures stimulated with untreated tyrosinase-peptide pulsed DC. In contrast, DC generated in the presence of IL-10 induced a markedly impaired proliferation of the T-cell line (Fig 3).

No proliferation was observed if a second unrelated mel-

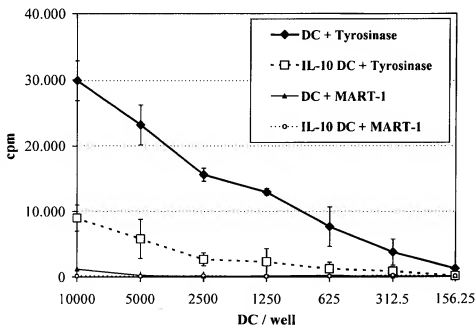


Fig 3. Inhibition of the proliferation of cytotoxic tyrosinase-specific CD8⁺ T cells by IL-10-treated DC. Tyrosinase-specific, cytotoxic CD8⁺ T cells were cultured with the specific tyrosinase peptide, either in combination with HLA-A2⁺ DC pretreated with IL-10 or untreated. In control experiments, the unspecific peptide MART-1 was added to the culture. Proliferation was measured by incorporation of [³H] TdR after 3 days of culture. The results represent one of three experiments.

nona-associated antigen MART-1 was added to the culture medium independently of the pretreatment of the DC with IL-10, indicating a peptide-specific proliferation of the CD8⁺ T-cell line.

Induction of peptide-specific anergy in a tyrosinase-specific CD8⁺ T-cell line. In the following experiments we evaluated whether tyrosinase-specific CD8⁺ T-cell lines were anergized by the stimulation with IL-10-pretreated DC. In a first coculture, the peptide-specific cytotoxic CD8⁺ T cells were cultured with autologous, IL-10-treated, or untreated (HLA.A2⁺) DC in combination with the specific tyrosinase peptide. Subsequently, T cells were rescued, cultured for 1 day (in some kinetic experiments up to 7 days; data not shown), and restimulated with mature, untreated DC in the presence of specific tyrosinase peptide in a second coculture (Fig 4).

After restimulation, a vigorous proliferative response was observed in CD8⁺ T cells precultured with untreated DC in the presence of the tyrosinase peptide. In contrast, coculture with IL-10-pretreated DC in the presence of the specific antigen induced a significant inhibition of the T-cell proliferation, indicating a tyrosinase-specific (melanoma-associated antigen-specific) anergy.

To show that the induction of anergy is antigen-specific, we stimulated the tyrosinase-specific CD8⁺ T-cell line during the first coculture with the unrelated peptide MART-1. After the second coculture, an unrestricted T-cell proliferation was observed, independent of a primary stimulation with untreated or IL-10-treated DC (Fig 4).

Additional control experiments were performed using untreated and IL-10-treated HLA-mismatched DC (HLA.A1⁺) stimulated with the tyrosinase peptide during the first coculture. After restimulation, an uninhibited proliferation of the CD8⁺ tyrosinase-specific T cells was demonstrated, indicating an

HLA-restricted antigen presentation and anergy induction (data not shown).

The addition of IL-2 (100 U/mL) to the second coculture reversed the state of peptide-specific anergy in the CD8⁺ T cells (Fig 4).

To analyze the cytokine pattern of the anergic, peptide-specific CD8⁺ T cells after restimulation, cytokines in the supernatants were detected by ELISA. The anergic T cells showed a markedly reduced secretion of the cytokines IL-2 and IFN- γ , but no production of IL-4 or IL-10. These data indicate a block in Tc1 cytokine production but no shift to a Tc2 pattern in the anergic T cells (Table 1).

Anergic CD8⁺ T cells fail to lyse melanoma cells. The release of immunosuppressive factors such as IL-10 has been described for many tumors, including malignant melanoma.⁸⁻¹⁹ To test whether this process might be one possible escape mechanism of tumor cells by inhibiting the stimulatory function of DC, we analyzed the cytotoxic function of the anergic, tyrosinase-specific CD8⁺ T cells after coculture with untreated or IL-10-treated DC. The cytotoxic activity was measured using the ⁵¹Cr-labeled tyrosinase-expressing and HLA.A2⁺ melanoma cells SK-MEL 28 as target cells. Tyrosinase-specific control and anergic CD8⁺ T cells were cocultured with the melanoma cells for 4 hours in effector-target ratios, as indicated (Fig 5).

The tyrosinase-specific CD8⁺ T cells, precultured with untreated DC, showed a strong cytotoxic activity at all effector: target ratios used, with up to 85% lysis of the target tumor cells (Fig 5). In contrast, after prior coculture with IL-10-treated DC, tyrosinase-specific anergic CD8⁺ T cells induced a significantly decreased lysis of the melanoma cells.

Anergy specificity was demonstrated by control experiments. No induction of the cytotoxicity was shown if the unrelated

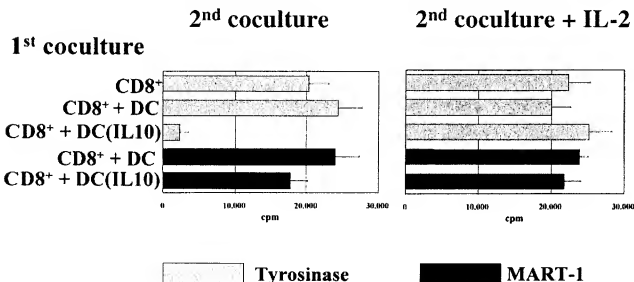


Fig 4. Induction of melanoma antigen-specific anergy in tyrosinase-specific, cytotoxic CD8⁺ T cells. In a first coculture, tyrosinase-specific HLA.A2⁺ CD8⁺ T cells were used as responder cells for HLA.A2⁺ DC additionally pretreated with IL-10 or untreated (cultured in complete medium alone). Cultures were set up in the presence of the specific tyrosinase peptide or an unspecific control peptide (MART-1). Subsequently, the T cells were rescued, cultured for 1 to 7 days in the presence of low levels of IL-2 (2 U/mL), and restimulated with mature HLA.A2⁺ DC during the second coculture. After 2 days, the proliferation was measured by [³H]TdR incorporation. The left bars show the specific response and the right bars show the T-cell response to IL-2 (100 U/mL). The results are representative for three experiments.

Table 1. Suppression of Cytokine Production of Anergic Tyrosinase-Specific CD8⁺ T Cells After Restimulation

First Coculture	Cytokine Production After Second Coculture (pg/mL)			
	IL-2	IFN- γ	IL-4	TGF- β
Tyrosinase-specific CD8⁺ T cells and				
DC	987 \pm 23	3,765 \pm 124	ND	ND
DC (IL-10)	1,102 \pm 76	4,087 \pm 218	ND	ND
DC + tyrosinase peptide	1,154 \pm 205	3,867 \pm 134	ND	ND
DC (IL-10) + tyrosinase peptide	105 \pm 20	258 \pm 87	ND	ND
DC + MART-1	1,287 \pm 156	3,841 \pm 434	ND	ND
DC (IL-10) + MART-1	1,087 \pm 187	4,187 \pm 128	ND	ND
DC [HLA.A1*] + tyrosinase peptide	1,376 \pm 231	4,283 \pm 349	ND	ND
DC (IL-10) [HLA.A1*] + tyrosinase peptide	1,498 \pm 327	3,762 \pm 297	ND	ND

Tyrosinase-specific CD8⁺ T cells were cocultured with untreated or IL-10-treated DC in combination with the specific tyrosinase peptide, with an unspecific peptide (MART-1), or without antigen during the first coculture. Additional control experiments were performed using HLA-mismatched (HLA.A1*) untreated and IL-10-treated DC and the specific tyrosinase peptide. After 2 days, the T cells were rescued, cultured for 2 days in the presence of low levels of IL-2 (2 U/mL), and restimulated with untreated DC and the specific tyrosinase peptide. Subsequently, the supernatants of the T-cell culture were harvested and assessed for IL-2, IL-4, IL-10, VTGF- β , and IFN- γ content by ELISA.

Abbreviation: ND, not detectable.

peptide MART-1 was added to the tyrosinase-specific CD8⁺ T cells during the primary culture, independent of a coculture with untreated or IL-10-treated DC (Fig 5).

To show the HLA-dependent restriction of the anergy induction, control experiments with HLA-mismatched (HLA.A1*) untreated and IL-10-treated DC pulsed with the tyrosinase peptide during the primary culture were performed. The stimulated tyrosinase-specific CD8⁺ T cells demonstrated an uninhibited lysis of the tyrosinase-expressing, HLA.A2* melanoma cells, independent of the use of untreated or IL-10-treated, HLA.A1* DC (data not shown).

Similar results were observed if peptide-pulsed mature DC were used as target cells. Coculture of anergic tyrosinase-specific CD8⁺ T cells with tyrosinase-pulsed DC in a ⁵¹Cr-

release assay led to a markedly reduced lysis of the target cells compared with control tyrosinase-specific CD8⁺ T cells. Peptide-specificity was demonstrated by the use of DC pulsed with the unrelated peptide MART-1 (data not shown).

DISCUSSION

The release of immunosuppressive factors such as IL-10 has been described for many tumors, including malignant melanoma.⁹⁻¹⁹ This immunologic process might be a mechanism of tumor cells to inhibit immune surveillance by converting DC from potent stimulatory cells of the immune system to tolerogenic APC.

In the present study, we investigated the effect of human IL-10-treated DC on the properties of cytotoxic CD8⁺ T cells

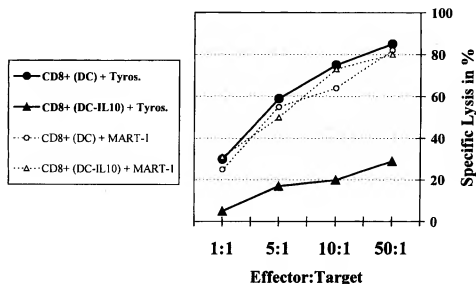


Fig 5. Anergic tyrosinase-specific CTL fail to lyse tumor cells. Control (precultured with untreated DC) and anergic (precultured with IL-10-treated DC) tyrosinase-specific HLA.A2* CD8⁺ T cells were cocultured with the HLA.A2* tyrosinase-expressing melanoma cell line SK-MEL 28 in a ⁵¹Cr-release assay for 4 hours. Experiments with specific CD8⁺ T cells cocultured with MART-1 during the primary culture served as controls. Various effector:T-cell ratios were used in the experimental setting. The percentage of specific lysis was calculated from the average of triplicates as 100 \times (⁵¹Cr-release into supernatant - spontaneous release)/(total release in detergent - spontaneous release). All synthetic peptides were tested for nonspecific lysis of target cells in the absence of CTL.

that are known to be involved in tumor rejection. We demonstrate that IL-10-treated DC induce an alloantigen-specific anergy in naive and activated CD8⁺ T cells and a tyrosinase-specific anergy in cytotoxic CD8⁺ T cells, resulting in a failure to lyse melanoma cells.

The escape mechanism of tumor cells regulated by the secretion of IL-10 may be due either to inhibition of recognition by immune cells or to inhibition of destruction of tumor cells by effector cells. In support of the first possibility, it was shown that IL-10 downregulates MHC class I expression by tumor cells and prevents their lysis by cytotoxic T cells.^{34,35} Although in these models a decreased expression of MHC class I molecules is described, significant residual levels of class I antigens that remain on the tumor targets argue against this alteration of tumor cells being the sole mechanism responsible for resistance to CTL lysis.^{34,35}

Alternatively, IL-10 might inhibit the antigen-presenting function of APC-like DC or macrophages and thus prevent a T-cell-mediated antitumor response. Experiments have shown that IL-10 downregulates the stimulatory capacity of APC such as macrophages and monocytes, but not that of B cells. This inhibitory influence of IL-10 is due to the downregulation of MHC class II molecules and the costimulatory molecules B7-1/-2 and intercellular adhesion molecule (ICAM-1).^{21,22,24,25} Furthermore, IL-10 reduces the release of a variety of inflammatory cytokines by monocytes/macrophages, including IL-1, IL-6, IL-8, TNF- α , and GM-CSF.^{23,26-28}

We recently demonstrated that IL-10-treated human DC induce an alloantigen- or peptide-specific anergy in CD4⁺ T cells, if IL-10 was added to immature DC.³¹ The pretreatment with IL-10 results in a reduced expression of the costimulatory molecules CD58 and CD86 and MHC class II molecules.³¹ The induction of anergy in various populations of T cells in this system might be due to a lack of costimulatory molecules and can be partially overcome by stimulatory CD28 MoAbs. Additionally, analysis of the supernatants of the IL-10-treated DC demonstrated an inhibited production of the proinflammatory cytokines IL-1 β , IL-6, and TNF- α and a lack of IL-12 production by the DC.

In our present study, we demonstrate that IL-10-treated human DC are able to induce a state of antigen-specific anergy also in CD8⁺ T cells. In the case of the tyrosinase-specific CD8⁺ T cells, a significantly decreased lysis of melanoma cells was observed after coculture with the anergic CD8⁺ T cells and the tumor cells in a ⁵¹Cr-release assay. Analysis of the cytokine pattern of the anergic T cells showed a diminished secretion of IL-2 and IFN- γ . Because IL-10-treated DC are known to have a reduced expression of costimulatory molecules such as CD86 and show a reduced production of IL-12, this might result in a defect to activate effector function of CTL characterized by the inhibition of IL-2 and IFN- γ production.

As a consequence, our findings support earlier data showing that production of IL-10 by tumor and/or tumor-infiltrating lymphoid cells might serve as a mechanism for tumor-induced anergy. This induction of anergy was described in various tumor models, including malignant melanoma.^{20,36} In these systems, IL-10 can act directly on the properties of cytotoxic CD8⁺ T cells as shown as a significantly reduced lysis of murine lymphoma cells in an *in vitro* model.³⁶ On the other hand, IL-10

is able to modulate the stimulatory characteristics of the APC by altering the surface expression of various MHC class I/II, costimulatory molecules, or the pattern of the cytokines secreted.²¹⁻²⁸ Investigations of human DC of responding or progressing melanoma metastases demonstrated a markedly increased production of IL-10 in tumor cells of progressively growing melanomas.²⁰ Furthermore, in a costimulation-dependent anti-CD3 tolerance assay, DC of the progressive tumor cells but not of the responding cells induce a state of antigen-specific anergy in cocultured T lymphocytes.²⁰

Additional studies have shown that IL-10 inhibits tumor antigen presentation by epidermal antigen-presenting cells in a murine squamous cell carcinoma model,²⁹ and in a murine plasmacytoma model it was demonstrated that, in the initiation but not in the effector phase of the immune response, IL-10 prevents DC accumulation in the tumor and inhibits rejection of the tumor if IL-10 was simultaneously expressed in the GM-CSF-transfected tumor cells.³⁷ Furthermore, IL-10 transgenic mice, in which the expression of IL-10 was expressed under the control of the IL-2 promoter, were unable to limit the growth of Lewis lung tumor cells.³⁸ The injection of anti-IL-10 antibodies significantly reduced the tumor progression, demonstrating a direct effect of IL-10 transgene to IL-10 action.³⁸

Paradoxically, in some experimental models, IL-10 has an immunostimulatory activity. Systemic injection of IL-10, transfection of tumor cells with IL-10, or the high physiological expression of IL-10 in certain tumors resulted in an increased immunogenicity and rejection of the tumor cells.³⁹⁻⁴³

These contrasting results might be due to the different tumor models used, the varying amounts of IL-10 used, and the different forms of IL-10 (virus IL-10 v cellular IL-10) applied. It was demonstrated that the antitumor effect of IL-10 was dose-dependent and that only very high levels of IL-10 were effective in tumor rejection.⁴⁰⁻⁴³ Furthermore, the amount of IL-10 required for immunosuppression may change for different tumors.

In our model, we demonstrate that a pretreatment of human DC with IL-10 induces a state of antigen-specific anergy in cytotoxic CD8⁺ T cells. When a tyrosinase-specific CD8⁺ T-cell line was used, the anergic T cells failed to lyse melanoma cells. Therefore, the secretion of IL-10 in the environment of tumor cells might be one mechanism of tumors to inhibit immune surveillance by reversing the properties of human DC from potent stimulatory cells of the immune system to tolerance of inducing cells.

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ATTACHMENT 2

Pisa, et al.

Selective expression of interleukin 10, interferon γ , and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies

(mRNA/polymerase chain reaction/T-cell receptor)

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ABSTRACT The variable clinical response seen with most cancer immunotherapy suggests that there is a large interindividual variation in immunologic response to tumors. One of the key functional parameters of an immune response is the local production of cytokines. As a method to survey the immune status of tumor-infiltrating cells, we have investigated the constitutive expression of cytokine mRNA in biopsies from epithelial ovarian carcinomas by using a PCR-assisted mRNA amplification assay. Using a set of cytokine-specific primers for 10 different cytokines, we have found selective expression of interleukin 10 (IL-10), granulocyte-macrophage colony-stimulating factor, and interferon γ mRNA in ovarian tumor tissue as compared to normal ovaries and ovarian tumor cell lines. Such differences could not be explained by the extent of T-cell infiltration, since comparing samples with the same intensity of T-cell receptor (TCR) constant region α -chain product from the tumor and normal biopsies demonstrated different cytokine patterns. No IL-2 gene expression was detected in the tumor biopsies. IL-2 mRNA, however, became expressed after stimulation of the tumor-derived cells via the CD3 molecule but not after growth in recombinant IL-2 alone. Using the same methodology, we also analyzed the TCR variable region β -chain gene repertoire. No restriction or biased expression of these genes was observed.

The variable clinical response seen with most cancer immunotherapy suggests that individual patients have very different immunological mechanisms involved in the pathophysiology of their specific malignancy (1, 2). These can include important differences in the existence, nature, and distribution of tumor antigens on the malignant cells, as well as the specific immune response to the tumor. Analysis of tumor-infiltrating inflammatory cells revealed various numbers of cytotoxic and helper T cells, natural killer (NK) cells, macrophages, and neutrophils (3–5). Freshly isolated tumor-infiltrating lymphocytes (TILs) are functionally deficient as measured by their proliferation and cytotoxicity (6, 7). Addition of recombinant interleukin 2 (rIL-2), however, renders these cells lytic *in vitro* (8) and capable of antitumor activity in adoptive transfer studies (1). These data suggest, therefore, that the failure of the immune system to recognize and destroy cancer cells may be in part a result of insufficient immunological activation. The mechanism underlying decreased immunological reactivity is not known and could involve suppression effectuated by immunologically active cells, energy due to aberrant presentation of antigen, and secretion of suppressor molecules from the tumor cells.

One of the key functional parameters of an immune response is the local production of cytokines. Under physiological conditions, these are transiently produced by more than one cell type and provide short-range signaling within the tissue. Depending on the sequence of their action, they may act either synergistically or antagonistically on multiple types of target cells (9). Recognizing these intricate interactions, attention is shifting from cytokine action on individual cells to cytokine action in tissues.

As a method to survey the immune status of tumor-infiltrating cells, we have investigated the constitutive expression of cytokine mRNA in biopsies from epithelial ovarian carcinomas by using a PCR-assisted mRNA amplification assay. Using a set of cytokine-specific primers for 10 different cytokines, we have found a selective expression of IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon γ (IFN- γ) mRNA in the ovarian tumor tissue as compared to normal ovaries and ovarian tumor cell lines. By the same methodology, experiments were also performed to correlate the cytokine mRNA pattern of each tumor biopsy with its T-cell receptor variable region β -chain (TCR V β) gene repertoire.

MATERIALS AND METHODS

Biopsy Samples. Ovarian tumor biopsy specimens were obtained from 11 patients with advanced epithelial ovarian carcinomas, histologically classified according to World Health Organization criteria (10). All patients underwent primary extensive debulking surgery.

Biopsy specimens from normal ovaries were obtained in patients laparotomized for nonmalignant gynecological disorders. Three of these women were postmenopausal. Informed consent according to the Helsinki agreement was obtained from the patients before surgery.

Immediately after surgical removal of the first tumor specimen, biopsies were trimmed, frozen in CO₂-chilled isopentane, and kept at -70°C until analysis.

Tumor Cell Lines. Tumor tissue was minced with a scalpel and digested with collagenase type S (1 mg/ml), DNase (200 μ g/ml), and hyaluronidase (100 units/ml) (all Sigma). Plastic adherent cells were cultured in Dulbecco's modified Eagle's medium (GIBCO), supplemented with L-glutamine (300 μ g/ml), penicillin (100 units/ml), streptomycin (100 μ g/ml), and 10% fetal calf serum (GIBCO). Temporary selection medium

Abbreviations: IL, interleukin; rIL, recombinant IL; IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TCR V and C genes, T-cell receptor variable and constant region genes; TIL, tumor-infiltrating lymphocyte; DIG, dUTP-11-dioxigenin.

(MEM C-VAL; GIBCO) was used to prevent fibroblast growth.

PCR-Assisted mRNA Amplification. RNA preparation. Total RNA preparation was performed essentially as described (11). In brief, 50–150 mg of frozen tissue was lysed in a proportionate volume (0.5–1.5 ml) of solution, consisting of 4 M guanidine thiocyanate (Fluka), 25 mM sodium citrate (pH 7), 0.5% sodium *N*-laurylsarcosinate (Fluka), and 100 mM 2-mercaptoethanol (Sigma). To each 1 ml of lysate was subsequently added 0.1 ml of 2 M sodium acetate, 1.0 ml of water-saturated phenol (Fluka), and 0.4 ml of chloroform/isomyl alcohol (49:1); ingredients were thoroughly mixed by inversion after the addition of each reagent. The final suspension was shaken for 10 sec and chilled on ice for 15 min. Samples were spun at $10,000 \times g$ for 20 min at $+4^\circ\text{C}$, and the aqueous phase was transferred to a clean Eppendorf tube; RNA was precipitated in an equal volume of 2-propanol at -20°C for 60 min. Precipitates were pelleted at $10,000 \times g$ at $+4^\circ\text{C}$, redissolved in lysis solution, and ethanol precipitated overnight at -20°C ; they were subsequently washed twice in 75% ethanol. Air-dried pellets were resuspended in 0.1–0.3 ml of RNase-free water.

First-strand cDNA synthesis. RNA was denatured for 5 min at 70°C and then chilled on ice. First-strand cDNA synthesis was performed from 5–10 μg of RNA at 40°C for 45 min in a final vol of 0.1 ml: 50 μl of denatured RNA, 20 μl of 5 \times buffer (BRL), 7.5 μl of 100 mM dithiothreitol (BRL), 10 μl of dNTP (dATP, dCTP, dGTP, and dTTP (5 mM each; Pharmacia), 2.5 μl of RNasin (40 units/ μl ; Promega), 5 μl of 1 M random hexamer primers (Pharmacia), 5 μl of Moloney murine leukemia virus reverse transcriptase (200 units/ μl ; BRL).

Tubes were afterwards heated for 5 min at 95°C . cDNA from each sample was synthesized in one tube and then divided into separate tubes for the PCR.

Cytokine cDNA amplification. Forty microliters of PCR mixture was added to 10 μl of first-strand cDNA. PCR mixture contained 5 μl of $10\times$ buffer [100 mM Tris-HCl/500 mM KCl/0.1% (wt/vol) gelatin, pH 8.3], 5 μl of 20 mM MgCl_2 , 8 μl of dNTP (1.25 mM each; Pharmacia), 11.75 μl of sterile water, 5 μl of each primer (10 μM), and 0.25 μl of *Taq* polymerase (Cetus). The reaction mixture was amplified with a Perkin-Elmer thermal cycler for 30 cycles. The temperature profile used was 94°C for 1 min for denaturation, 58°C for 1 min for annealing, and 72°C for 1 min for primer extension. PCR products were separated on ethidium bromide-stained 1.6% agarose gel (Pharmacia). Lymphokine-specific primers were synthesized on a DNA synthesizer (Applied Biosystems). All primers were RNA specific and nonreactive with DNA. The following oligonucleotide 5' and 3' primer sequences were used: IL-1 α , GCCAATGACTCAGAGGAAGA and TCTCAGGCATCTCTTCAGC; IL-2, TGTCACGAGTGCAACCTCTG and CAATGGTGTGCTGTCTCATCAG; IL-3, CTCCTGTCTCAACTCTGTTG and AGGCTCAAAGT-CGTCTGTGT; IL-4, CCTCTGTCTCTCTGTCTGATG and CCGTTCACGGAATCGGATCA; IL-6, TGAACCTCTCTCTCACAAGC and ATCCACGATTGGAAGCATCCA; IL-10, CTGAGAACCAAGACCCAGACATCAAGG and CAATAAGGTTTCTCAAGGGCTGGGCT (12); tumor necrosis factor α (TNF- α), TGAGCATGCAAGCATGATC and TTATCTCTCAGCTCCAGCC; IFN- γ , TCTGCATCGTTT-TGGGTCT and CAGCTTTCGGAATCATCTC; GM-CSF, TGACAGGCTGCTGCTCTTG and CAAGCAG-AAGTCTCTCAGG; G-CSF, CAGGCCCCATGAAGC-TGAT and TATGAGTGTGCTCAAGCAG; β -actin, ATG-GATGATGATATCGCCGCG and CTAGAAGCATTTCCGGTGGAC.

Amplification of TCR V_β and constant region α -chain (C_α) cDNA. One microliter of cDNA was mixed with 19 μl of PCR mixture containing 0.5 μM (final concentration) TCR C_α -or

V_β -specific oligonucleotide. Primer sequences (13) were verified by analysis of RNA extracted from phytohemagglutinin-stimulated lymphocytes, which resulted in successful amplification of all TCR V_β genes (data not shown). The PCR profile used was denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles. Five microliters of the TCR C_α amplified product was separated on an ethidium bromide-stained 1.6% agarose gel and visualized in UV light. The V_β PCR amplified product was separated on a 1% agarose gel and blotted by alkali transfer to a nylon filter (Hybond-N; Amersham). A C_β -specific probe (14) was prepared as follows: 10 ng of plasmid cDNA was amplified (total vol, 50 μl) using primers 5' C_β -AGGACCT-GAAC and 3' C_β -GGGAGATCTCTGCTTCTGTATGG (final concentration, 0.5 μM each) and dNTP with a 2:1 ratio between dTTP and dUTP-11-digoxigenin (DIG) (Boehringer Mannheim). The PCR profile used was 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min for 30 cycles. Filters were hybridized overnight at 42°C with DIG-labeled probe and incubated with alkaline phosphatase-labeled antibody. Finally, membranes were developed using the chemiluminescent substrate 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) by autoradiography for 5–45 min according to the manufacturer's instructions (Boehringer Mannheim).

Statistical Analysis. Fisher's exact probability test was used for statistical analysis of cytokine gene expression.

RESULTS

Expression of cytokine and TCR V_β genes was analyzed in biopsies taken from 11 ovarian carcinomas by the PCR-assisted mRNA amplification assay. Biopsies from 8 healthy ovaries and 2 *in vitro* established ovarian carcinoma cell lines were included as controls.

T-Cell Infiltration. To estimate the degree of T-cell infiltration in the tumors and normal ovaries, the TCR C_α gene was analyzed by PCR. C_α was successfully amplified in all 11 tumor biopsies, with bands of high intensity, as detected on ethidium bromide-stained gels (Fig. 1A). In contrast, 3 of the 8 normal ovaries tested failed to express mRNA for TCR C_α (Fig. 1B). The intensity of the amplified product in these 5 biopsies varied. These results demonstrate T-cell infiltration in all tumor biopsies and in most, but not all, normal ovaries.

Cytokine mRNA Expression. Cytokine gene expression was detectable in 10 of the 11 tumor biopsies, in 5 of the 8 normal ovaries, and in both tumor cell lines (Table 1). To rule out the possibility that a negative result in some samples was due to

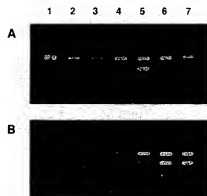


Fig. 1. TCR C_α gene expression in tumor biopsies and normal ovaries. cDNA from ovarian tumor biopsies (A) and normal ovaries (B) was amplified by using oligonucleotide primers specific for TCR C_α . The size of the amplification product is 600 base pairs (bp). (A) Lanes 1–7 correspond to patients 1–7 in Table 2. (B) Lanes 5–7 correspond to normal ovaries 1–3 from the same table.

Table 1. Cytokine mRNA expression in ovarian biopsies from cancer patients, healthy donors, and ovarian tumor cell lines

	IL-1 α	IL-2	IL-3	IL-4	IL-6	IL-10	TNF- α	IFN- γ	GM-CSF	G-CSF
Healthy donors ($n = 8$)	0	0	0	0	3	0	5	2	0	0
Cancer patients ($n = 11$)	8	0	1	0	7	10*	10	8*	8*	1
Tumor cell lines ($n = 2$)	2	0	0	0	2	0	1	0	0	1

*P values for IL-10, IFN- γ , and GM-CSF in the tumor biopsies compared to normal ovaries are 0.0001, 0.0500, and 0.0021, respectively.

inefficient transcription and/or failure of the PCR, mRNA for the β -actin gene was successfully amplified in all cases. Ten of 11 tumor biopsies expressed mRNA for IL-10 and TNF- α ; in 8 tumors, IL-1 α , IFN- γ , and GM-CSF were observed; 7 exhibited IL-6; and 1 biopsy also expressed mRNA for G-CSF (Fig. 2; see also Fig. 4A). In contrast to the tumor biopsies, none of the 8 normal ovaries expressed IL-10 or GM-CSF mRNA (Fig. 3A). Three of them expressed IL-6 mRNA, 5 expressed TNF- α , and 2 expressed IFN- γ mRNA. Of the 2 ovarian tumor lines (passages 2 and 20), cytokine mRNA for IL-1 α and IL-6 was detected in both, while TNF- α and G-CSF were expressed in 1 (Fig. 3B).

Cytokine expression analysis (Table 1) showed that the presence of IL-10 ($P = 0.0001$) and GM-CSF ($P = 0.0218$) mRNA was unique for the tumor biopsies. IFN- γ ($P = 0.0500$) gene expression was more frequent in the tumor biopsies but it was also present in healthy ovaries. Genes coding for IL-1 α , IL-6, TNF- α , and G-CSF were expressed in tumor biopsies, tumor lines, and/or healthy ovaries, while IL-2, -3, and -4 mRNA were not detectable in any of the biopsies tested. No consistent pattern was seen when comparing the pathological classification of tumors (7 endometrioid, 3 seropapillary, and 1 mucinous) with their cytokine profile (data not shown).

To analyze the potential of the T cells infiltrating the tumor biopsies to express IL-2, -3, and -4 mRNA, we polyclonally activated one tumor sample, available as an ascites, by an anti-CD3 monoclonal antibody. This sample expressed a typical cytokine profile observed among the tumor biopsies

(Fig. 4A). A 7-hr culture with the OKT3 antibody resulted in expression of all cytokines except IL-3 (Fig. 4B). However, cells cultured for 10 days with rIL-2 alone exhibited their original cytokine profile, without the expression of IL-2, -3, or -4, demonstrating that TCR stimulation was necessary for such expression (data not shown).

TCR V β mRNA Expression in Biopsies from Ovarian Carcinomas. Since the observed expression of cytokine mRNA indicated the presence of immunologically active cells, we also analyzed the T-cell repertoire in tumor and normal ovarian tissues. A representative Southern blot analysis of the PCR-amplified TCR V β gene is shown in Fig. 5. Results from the analysis of all 11 ovarian cancer biopsies and from 3 normal ovaries are summarized in Table 2. As can be seen for most tumors, there was a heterogeneity in the TCR V β repertoire. V β gene usage varied from very limited (biopsies 2, 3, 6, and 7) to a broad repertoire of TCR V β expression. The observed differences in the intensity of the signals indicated quantitative variation between different V β genes, as shown in Table 2 by + for a strong signal and by \pm for a weak signal. There was no biased usage of individual V β genes in the tumor biopsies, although V β 1 to -9 seemed to be more frequently expressed and V β 11, -15, and -20 were only detected in a minority of samples. The normal ovaries that demonstrated T-cell infiltration also had heterogeneous expression of TCR V β genes (Table 2). No correlation between cytokine expression and V β gene usage was observed (data not shown).

DISCUSSION

A major coordinating function in an immune response is executed by cytokines. Recent published data provide evidence that the pattern of cytokine expression in skin biopsies from patients with leprosy correlated with resistance or

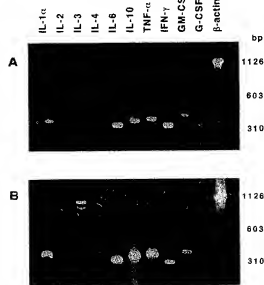


Fig. 2. Cytokine profile in tumor biopsies. Total RNA extracted from biopsy specimens was reverse-transcribed into cDNA. cDNA was amplified by PCR using cytokine mRNA-specific oligonucleotide primers. As an internal control of reverse transcription and PCR, β -actin mRNA was successfully amplified in all samples. (A and B) Cytokine gene expression in tumor biopsies 1 and 10 in Table 2, respectively. Bands >500 bp in lane IL-3 in B are larger than the expected IL-3 PCR product (425 bp) and are due to DNA contamination.

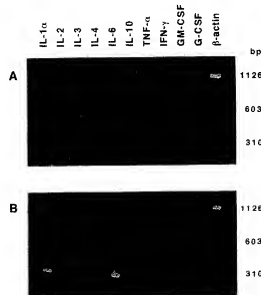


Fig. 3. Cytokine profile in normal ovaries and ovarian tumor cell lines. A representative cytokine profile from a normal ovary (ovary 3 in Table 2) is shown in A and one from an *in vitro* established ovarian tumor cell line (passage 20) is shown in B.

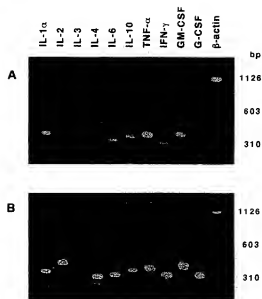


FIG. 4. Cytokine gene expression in ovarian tumor cells before and after OKT3 stimulation. An ovarian tumor from patient 4 in Table 2 available as an ascites and exhibiting a typical tumor cytokine profile was cultured (2×10^6 cells per ml) in RPMI medium with purified OKT3 (25 ng/ml) murine monoclonal antibody (American Type Culture Collection) for 7 hr. To reduce nonspecific stimulation, the serum source was excluded from the culture. Total RNA representing 5×10^4 cells from unstimulated (A) and stimulated (B) culture was used for amplification of each cytokine gene.

susceptibility to the infection (15). Furthermore, it was reported that the different functions of human CD4⁺ and CD8⁺ T-cell subsets can be discriminated and predicted by analysis of their cytokine profile (16). We now show that cytokine mRNA profiles of ovarian cancer biopsies differ from healthy ovaries. Comparison of an ovarian tumor cell line with cancer biopsy cytokine profiles revealed that the observed difference could not be accounted for by cytokine production of cancer cells only. Neither could the discrepancies be explained by the extent of T-cell infiltration, since comparing samples with the same intensity of TCR C α product from the tumor (Fig. 1A, lane 1) and normal (Fig. 1B, lane 5) biopsies demonstrated different cytokine patterns (Figs. 2A and 3A, respectively). Our results demonstrate that

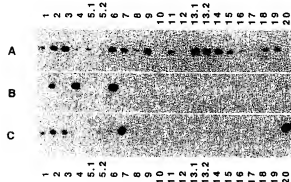


FIG. 5. Southern blot analysis of PCR-amplified TCR V β genes in ovarian tumor biopsies. Total RNA extracted from biopsy specimens was reverse-transcribed into cDNA by a random hexamer priming method. Oligonucleotide primers specific for 22 different TCR V β genes were used to amplify the cDNA in 35 cycles. Products were separated on 1% agarose gel, blotted, probed with DIG-labeled C β probe, and detected with alkaline phosphatase-labeled antibody and chemiluminescence AMPPD substrate. (A–C) Analysis of tumor biopsies from patients 1–3 in Table 2, respectively.

Table 2. Southern blot analysis of PCR-amplified TCR V β gene expression in ovarian tumor biopsies and normal ovaries

V β gene	Tumor biopsies										Normal ovaries					
	1	2	3	4	5	6	7	8	9	10	11	n = 11	1	2	3	n = 3
1	+	+	+	+	+	+	+	+	+	+	+	9	+	+	+	2
2	+	+	+	+	+	+	+	+	+	+	+	10	+	+	+	1
3	+	+	+	+	+	+	+	+	+	+	+	9	+	+	+	2
4	+	+	+	+	+	+	+	+	+	+	+	9	+	+	+	2
5.1	+	+	+	+	+	+	+	+	+	+	+	9	+	+	+	2
5.2	+	+	+	+	+	+	+	+	+	+	+	8	+	+	+	1
6	+	+	+	+	+	+	+	+	+	+	+	9	+	+	+	1
7	+	+	+	+	+	+	+	+	+	+	+	8	+	+	+	1
8	+	+	+	+	+	+	+	+	+	+	+	9	+	+	+	2
9	+	+	+	+	+	+	+	+	+	+	+	6	+	+	+	1
10	+	+	+	+	+	+	+	+	+	+	+	6	+	+	+	1
11	+	+	+	+	+	+	+	+	+	+	+	4	+	+	+	1
12	+	+	+	+	+	+	+	+	+	+	+	7	+	+	+	1
13.1	+	+	+	+	+	+	+	+	+	+	+	7	+	+	+	2
13.2	+	+	+	+	+	+	+	+	+	+	+	7	+	+	+	2
14	+	+	+	+	+	+	+	+	+	+	+	6	+	+	+	2
15	+	+	+	+	+	+	+	+	+	+	+	4	+	+	+	0
16	+	+	+	+	+	+	+	+	+	+	+	6	+	+	+	1
17	+	+	+	+	+	+	+	+	+	+	+	7	+	+	+	2
18	+	+	+	+	+	+	+	+	+	+	+	7	+	+	+	2
19	+	+	+	+	+	+	+	+	+	+	+	6	+	+	+	1
20	+	+	+	+	+	+	+	+	+	+	+	3	+	+	+	1
V β usage	21	3	9	20	20	5	2	18	19	17	20		19	6	6	

+, Strong signal; -, no signal; \pm , intermediate/weak signal.

some cytokines predominantly but not exclusively produced by T cells, such as IL-10, IFN- γ , and GM-CSF, are selectively expressed in the cancer lesions. Interestingly, mRNAs for other cytokines also believed to be of T-cell origin (IL-2, -3, and -4) were absent.

Previous *in vitro* studies also describe TNF- α , IFN- γ , and GM-CSF production in TILs expanded in rIL-2 and stimulated with autologous tumors (17–19). These and other studies (20) report a specific defect in IL-2 and IL-4 secretion by TILs, which might be overcome by phytohemagglutinin stimulation (21). Recently published data also demonstrate that generation of lymphokine-activated killer cells from peripheral blood lymphocytes of renal cancer patients may be down-regulated by coculture with autologous TILs or a cell-free supernatant from such cells (22). It was also suggested that defective IL-2 production might be one of the reasons for impaired immunity in cancer patients. Our results support this notion and further indicate that a defect in IL-2 and IL-4 gene expression is probably not at the transcriptional level, since stimulation of the TCR via the CD3 molecule induces expression of IL-2 and IL-4 mRNA. The data further indicate that the IL-2 deficiency cannot be overcome by mere rIL-2 stimulation, as the 10-day TIL culture exhibited the same cytokine profile as the original tumor and was also deficient in IL-2 mRNA. The observed lack of IL-2 expression therefore could result from a lack of TCR-mediated stimulation *in vivo*—e.g., resulting from an immunosuppression at the antigen presentation level. Such a defect could be in part explained by the observed expression of IL-10 in the cancer lesions, a cytokine that was first described as a cytokine synthesis inhibitory factor (23, 24). Recently, it was also demonstrated that IL-10 strongly inhibits antigen-specific T-cell proliferation through down-regulation of the major histocompatibility complex class II expression and in that way inhibits the antigen presenting capacity of monocytes (25, 26). However, the true role of

IL-10 in this context remains to be analyzed with the help of, e.g., blocking antibodies or antisense RNA constructs.

Certain cytokine expression in normal ovaries is not surprising, since, e.g., IL-1 and TNF- α were implicated as putative intraovarian regulators (27). Normal ovaries are known to be infiltrated by T lymphocytes (27), as confirmed by our data, where the majority of normal ovaries expressed C α mRNA. They exhibited, however, a different cytokine profile as compared to tumor biopsies, with a lack of IL-10 and GM-CSF expression and seldom expressing IFN- γ . We therefore conclude that the difference in cytokine expression between tumor and normal ovarian tissue cannot simply depend on a quantitative difference in T-cell infiltration.

Since the majority of TILs are believed to be of the CD3 $^{+}$ CD4 $^{+}$ /CD8 $^{-}$ phenotype (3, 28) the question that naturally arises is whether T-cell subset predominantly produces these cytokines. Murine CD4 $^{+}$ T cells have been divided according to their patterns of cytokine production (29, 30). There is now evidence that even human type 1 CD4 $^{+}$ cells produce IL-2, IFN- γ , and GM-CSF (16, 31). The human T-cell subset with helper activity for B cells, corresponding to the murine T H 2 cells, produces IL-4, IL-5, and GM-CSF. The human CD8 $^{+}$ population can also be divided into subsets: CD8 $^{+}$ cytotoxic cells (type 1) that produce mainly IFN- γ and IL-10 and CD8 $^{+}$ suppressor cells (type 2) that produce IL-4 (16). In the present study, the cytokine profile of individual T-cell clones derived from the tumor biopsies has not been addressed. Therefore, it is unclear to what extent the same subdivision of T-cell subsets in infectious diseases is also valid for the TILs. If so, however, then the predominant T-cell subset in the tumor biopsies would correspond to a type 1 CD8 $^{+}$ T cell, with no production of IL-4 but with significant amounts of IL-10, GM-CSF, and IFN- γ .

Regarding the possibility that γ/δ T cells are responsible for the observed cytokine expression, the phenotype of fresh noncultured tumor-infiltrating cells was analyzed by cytometry. Only 1–3% of the CD3 $^{+}$ cells expressed the γ/δ TCR (data not shown), not exceeding what is usually observed in peripheral blood. Taking into account the sensitivity of PCR methodology, this does not entirely rule out the fact that γ/δ T cells could be responsible for the observed cytokine expression, particularly as they are known to produce several cytokines, including IL-2, IL-4, IFN- γ , and GM-CSF (32). Even the contribution of CD3 $^{-}$ NK cells as well as activated macrophages to the observed cytokine profile should also be considered, since these were shown to produce IL-10 (24); however, their presence in ovarian malignancies is rare (5).

Little information exists on the T-cell repertoire of TILs in humans, although limited expression of TCR V α genes was reported in human melanomas (33). The majority of tumors in our study demonstrated heterogeneous expression of a broad TCR V α gene repertoire, but four of the tumor biopsies exhibited restricted TCR V α usage, with only two to five V α genes expressed (Table 2). The finding of relatively restricted V α expression in two of the normal ovaries may indicate, however, that this selectivity is tissue specific rather than characteristic of ovarian tumors.

Immunotherapy offers medical treatment of great specificity. However, the unique nature of the host-tumor relationship and the unfavorable risk/benefit ratio of the treatment are arguing against its indiscriminate application. Precise diagnostic assays able to describe the specific pathophysiology in individual patients could identify the "likely to respond" group of patients. Clinical efficacy in immunotherapy also requires determination of the optimal dose for individual patients. Our results demonstrate specific cytokine mRNA profiles in ovarian tumor biopsies, which

might be of prognostic significance for immunotherapy. The possibility of using a fine needle biopsy technique in conjunction with PCR methodology to monitor the "immune status" of tumor-infiltrating cells might facilitate the clinical use of such treatments. Furthermore, selective blocking of certain cytokines locally produced in the tumor, such as IL-10, might offer new immunotherapeutic principles against cancer.

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ATTACHMENT 3

Smith, et al.

Short Communication

Production of Interleukin-10 by Human Bronchogenic Carcinoma

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Interleukin-10 (IL-10) is a recently characterized cytokine with suppressive activity against various aspects of the cellular immune response. Our laboratory has previously demonstrated that another anti-inflammatory cytokine, IL-1 receptor antagonist (IRAP) is produced and secreted by human bronchogenic carcinomas. We speculated that tumor production of IRAP may mitigate host responses and confer increased tumor viability. In this study, we investigated the capacity of human bronchogenic tumors to produce IL-10 as another possible mechanism to attenuate host defenses. We found increased levels of antigenic IL-10 in tissue homogenates of human bronchogenic carcinomas compared with normal lung tissue (13.69 ± 2.87 versus 5.84 ± 0.84 ng/mg total protein). Immunohistochemical staining of tumors illustrate primary localization of antigenic IL-10 to individual tumor cells. Analysis of supernatants of several unstimulated human bronchogenic cell lines in vitro demonstrated the ability of tumor cells to constitutively produce IL-10. Functional studies of mononuclear cells, cultured in the presence of conditioned medium from a bronchogenic cell line, demonstrated their increased tumor necrosis factor and IL-6 production with the addition of neutralizing antibodies to IL-10. These findings demonstrate that human broncho-

genic carcinomas elaborate functional IL-10, which may significantly impair immune effector cell function and enable the tumor to evade host defenses. (Am J Pathol 1994; 145:18-25)

Human bronchogenic carcinoma displays an extremely aggressive clinical course and represents the leading cause of malignancy-related mortality in the United States.¹ This behavior may reflect an increased capacity to evade detection and containment by host immune responses. Potential mechanisms that tumors may use to avoid host defenses include the expression of antigens that lack sufficient immunogenicity, the down-regulation of tumor cell surface major histocompatibility complex (MHC) molecules, rapid growth kinetics that may produce resistant cell lines, and the masking or shedding of tumor antigens.² Finally, tumors may also secrete factors that directly suppress inflammatory responses and specific immune cell functions.²

Interleukin-10 (IL-10) is a recently characterized cytokine that demonstrates varied immunosuppressive bioactivity. Since its initial isolation by Mosmann et al^{3,4} in 1988, investigations have elucidated many of the immunological properties of this cytokine. Originally identified as a product of CD4⁺ T cells, IL-10 is also produced by monocytes, macrophages, B cells, certain populations of CD8⁺ T cells, and Epstein-Barr Virus (EBV)-transformed lymphoblastoid cells lines.⁵⁻⁸ Recent work has

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demonstrated that keratinocytes may also elaborate IL-10.⁹ A particularly interesting finding is that a previously uncharacterized open reading frame, BCRF-1, in the EBV genome bears striking homology with IL-10.^{6,10} Functional studies reveal that IL-10 has profound effects on monocytes, resulting in alterations in cell morphology and cytotoxicity, down-regulation of the expression of MHC class II antigens, and inhibition of proinflammatory cytokine production.¹¹⁻¹⁵ Furthermore, IL-10 also exerts direct effects on the growth and function of T cells, B cells, and mast cells.^{4,16-20} These specific actions result in the capacity for IL-10 to attenuate a wide range of effector immune responses, including T cell cytokine production and antigen-specific proliferation, B cell immunoglobulin synthesis, and the elaboration of tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) by natural killer cells.^{19,21-26} IL-10 may play an important role in homeostasis under normal circumstances and speculation has arisen that IL-10 biology may also have important consequences for certain infectious diseases, organ transplantation, immune tolerance, and cancer immunotherapies.

Previous work from this laboratory has demonstrated the capacity of human bronchogenic tumors to elaborate significant quantities of another immunosuppressive cytokine, IL-1 receptor antagonist protein (IRAP).²⁷ We have proposed that tumor secretion of IRAP may impair host responses and result in an increased ability of tumors to evade host defenses. Our earlier findings led us to further examine the immunosuppressive capacities of tumors and to postulate that bronchogenic carcinomas may also produce and secrete IL-10 as an additional means of attenuating normal host immune responses.

In this report we describe increased antigenic levels of IL-10 in homogenates of human bronchogenic carcinomas compared with normal lung tissue. Immunohistochemical staining of tumor tissue graphically localizes antigenic IL-10 to individual tumor cells, suggesting that the bronchogenic carcinomas rather than any infiltrating mononuclear cells are the primary source of increased IL-10 in tissue homogenates. Analysis of supernatants of human bronchogenic carcinomas maintained *in vitro* under unstimulated conditions demonstrate measurable quantities of IL-10 and confirm the capacity of tumor cells to produce and secrete IL-10. Finally, functional studies of mononuclear cells cultured in the presence of conditioned medium from a tumor cell line demonstrate increased mononuclear cell

IL-6 and TNF- α production with the addition of neutralizing antibodies to IL-10.

Materials and Methods

Tissue Samples

Lung tissue specimens were obtained in a prospective fashion from 58 consecutive patients undergoing thoracotomy for resection of suspected primary bronchogenic carcinoma. Two specimens were obtained from each patient; one from the tumor and one from an area of normal lung distal to the tumor. After recovery from the operating room, tissue samples were promptly homogenized and processed for protein isolation. Additional specimens were fixed in 4% paraformaldehyde for 24 hours before transfer to 70% ethanol and subsequent paraffin embedding. Final pathological diagnoses were determined by review of specimen slides by university hospital pathologists. Specimens of squamous cell carcinomas and adenocarcinomas were included in our study. Those of mixed cellularity or other pathological histology were excluded, leaving 47 specimens to be included in our evaluation.

Reagents

Polyclonal anti-human IL-10-, IL-6-, and TNF- α -specific antiserum were produced by the immunization of rabbits with human recombinant IL-10 (Pepro Tech Inc., Rocky Hill, NJ), IL-6 (R&D Systems, Minneapolis, MN), and TNF- α (Genentech, San Francisco, CA), respectively, in multiple intradermal sites with complete Freund's adjuvant. The specificity of each of these resulting antibodies was assessed by Western blot analysis against human recombinant IL-10, IL-6, and TNF- α . Antibodies were specific in our sandwich ELISA without cross-reactivity to a panel of 12 human recombinant ILs, including IL-1 α , IL-1 β , IRAP, IL-2, IL-4, IFN- γ , and members of the C-C or C-X-C chemokine family.

Cytokine ELISA

Antigenic IL-10, TNF- α , and IL-6 were quantitated using a modification of a double ligand method as previously described.²⁸ Briefly, flat-bottom 96-well microtiter plates (Nunc Immuno-Plate 1 96-F) were coated with 50 μ l/well of the appropriate polyclonal antibodies (1 ng/ μ l in 0.6 M NaCl, 0.26 M H₃BO₃, and 0.08 N NaOH, pH 9.6) for 24 hours at 4 C and then washed with phosphate-buffered saline (PBS), pH

7.5, 0.05% Tween 20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 60 minutes at 37°C. Plates were rinsed three times with wash buffer and diluted (neat and 1:10) and samples (50 µl/well) were added, followed by incubation for 1 hour at 37°C. Plates were washed three times and 50 µl/well of biotinylated polyclonal rabbit or the appropriate anti-human IL-10, IL-6, or TNF-α antibodies (3.5 ng/µl in PBS, pH 7.5, 0.05% Tween 20, and 2% fetal calf serum added and plates incubated for 45 minutes at 37°C.

Plates were washed three times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates incubated for 30 minutes at 37°C. Plates were washed three times and chromogen substrate (Bio-Rad Laboratories, Richmond, CA) added. The plates were incubated at room temperature to the desired extinction and the reaction terminated with 50 µl/well of 3 M H₂SO₄ solution. Plates were read at 490 nm in an automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Standards were 1/2 log dilutions of recombinant IL-10, IL-6, and TNF-α from 100 ng to 1 pg/ml (50 µl/well). This ELISA method consistently detected specific cytokine concentrations in a linear fashion greater than 50 pg/ml. All tissue homogenate specimens were run in parallel for total protein content (Pierce, Rockford, IL).

Immunohistochemical Localization of Antigenic IL-10

Paraffin-embedded tissue was processed for immunohistochemical localization of IL-10 protein using a modification of our previously described technique.^{27,29} Briefly, tissue sections were dewaxed with xylene and rehydrated through graded concentrations of ethanol. Tissue nonspecific binding sites were then blocked using normal goat serum (BioGenex, San Ramon, CA). Tissue sections were then washed and incubated with optimal concentrations of rat monoclonal (subclone JES3-9D7; PharMingen, San Diego, CA) antibodies to human IL-10 or equivalent concentrations of the same subclass rat IgG (rat IgG1; PharMingen). The tissue sections were washed and then incubated for 60 minutes with secondary goat anti-rat biotinylated antibodies (BioGenex). The tissue sections were then washed twice in TRIS-buffered saline and incubated with alkaline phosphatase conjugated to streptavidin (BioGenex). Fast Red (BioGenex) reagent was used for chromogenic localization of IL-10 antigen. After optimal color development, tissue sections were immersed in sterile

water, counterstained with Mayer's hematoxylin, and coverslipped using an aqueous mounting solution.

Tumor Cell Culture Supernatants

The human bronchogenic carcinoma cell lines A549, A427, and Calu-6 (ATCC, Rockville, MD) were cultured in 35-mm tissue culture plates under standard conditions and overlaid with 1 ml of recommended medium. RPMI 1640 medium with 100 U/ml penicillin, 100 mg/ml streptomycin, 25 mM HEPES, 1 mM L-glutamine, and 10% fetal calf serum was used for A549 cells, whereas the A427 and Calu-6 lines were maintained in Eagle's minimum essential medium with nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum.

Cell cultures were grown to 90% confluence, washed twice with appropriate medium, then overlaid with fresh medium (1 ml) and allowed to incubate for an additional 24 hours. Trypan blue staining demonstrated >90% viability. Conditioned medium was harvested and centrifuged 15 minutes at 600 g to remove any cellular debris then frozen at -20°C until further analyzed by specific ELISA. An additional, larger quantity of A549-conditioned medium for mononuclear cell studies was produced by culturing cells in 225-cm² flasks until confluent then washing twice and overlaying cells with fresh medium overnight before harvesting, centrifuging, and freezing the resulting conditioned medium.

Mononuclear Cell Isolation and Culture

Human peripheral blood mononuclear cells were isolated from healthy volunteers. Briefly, heparinized venous blood was obtained then processed by Ficoll-Hypaque centrifugation. Mononuclear cells were then resuspended in complete medium and washed three times. After counting cells with a hemacytometer using light microscopy, cells were washed again and resuspended at a concentration of 5×10^6 cell/ml in conditioned medium from A549 tumor cells in culture. Mononuclear cells were then cultured for 24 hours in 35-mm well plates alone and with 1:10 concentrations of either rabbit anti-human IL-10 or control sera. Supernatants were harvested, centrifuged, and frozen until analyzed by specific ELISA for IL-6 and TNF-α content.

Statistical Analysis

Data were analyzed by a Macintosh IIx computer using the Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA). Data were expressed as

mean \pm SEM and compared using a Student's two-tailed *t*-test. Data were considered statistically significant if *P* values \leq 0.05.

Results

We postulated that a potential mechanism that human bronchogenic carcinomas may use to attenuate host immune responses is the production and secretion of the immunoregulating cytokine IL-10. To test our hypothesis, we first analyzed and compared tissue homogenates of normal lung and lung tumors for the presence of IL-10. Our initial findings demonstrated 2.3-fold elevations in the levels of antigenic IL-10, normalized to total protein (TP), from bronchogenic carcinoma tissue compared with normal lung tissue homogenates ($P < 0.005$). As shown in Figure 1, tumor tissue-derived IL-10 was 13.69 ± 2.87 ng/mg TP compared with 5.84 ± 0.84 ng/mg TP for normal lung tissue ($n = 47$ patients). Subgroup analysis by specific histology revealed no difference in elevations of

antigenic IL-10 for squamous carcinomas ($n = 21$) and adenocarcinomas ($n = 26$) with IL-10 levels of 15.41 ± 3.70 ng/mg TP and 12.29 ± 4.28 ng/mg TP, respectively. IL-10 content varied within normal and tumor tissue, as demonstrated by scattergram in Figure 1, B, with some degree of overlap.

To determine the cellular source of antigenic IL-10 in tumor tissue we used immunohistochemistry. Samples of normal lung and lung tumor tissue were fixed and immunostained with specific monoclonal antibodies for IL-10. Our results demonstrated that immunoreactive IL-10 in tumor tissue was primarily localized to individual tumor cells in a heterogeneous staining pattern for all tumors studied (Figure 2, C and D). In contrast, samples of normal lung tissue demonstrated no staining for IL-10 (Figure 2, B). Staining with control rat IgG demonstrated the absence of significant nonspecific staining of tumor tissue (Figure 2, A). In addition, stains using HAM56 (Enzo Diagnostics, Inc., Farmingdale, NY), a murine monoclonal antibody against human mononuclear cells,

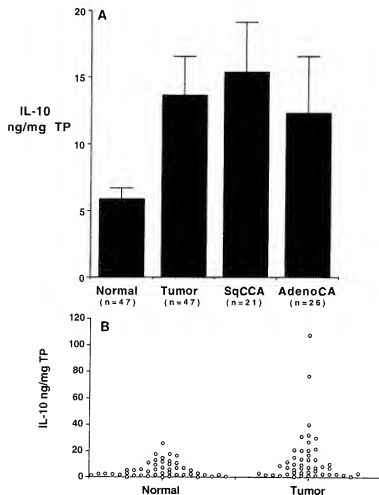


Figure 1. A: Antigenic determinations of IL-10 in tissue homogenates for normal lung and human bronchogenic carcinoma tumor tissue ($n = 47$). Normal lung tissue IL-10 content = 5.84 ± 0.84 ng/mg TP. Tumor tissue IL-10 content = 13.69 ± 2.87 ng/mg TP. Tumor subgroup analysis by final histological diagnosis also included demonstrating squamous cell carcinoma ($n = 21$) and adenocarcinoma ($n = 26$) with IL-10 levels of 15.41 ± 3.70 and 12.29 ± 4.28 ng/mg TP, respectively. B: Scattergram demonstrating distribution of IL-10 content of normal and tumor specimens.

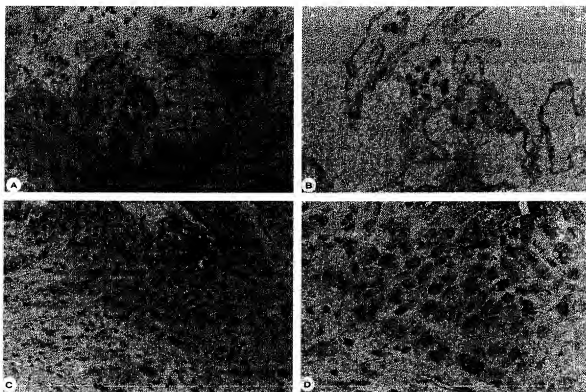


Figure 2. Representative photomicrographs of immunohistochemical staining of IL-10 in human bronchogenic carcinoma. Demonstrates positive staining with specific anti-IL-10 antibody at $\times 200$ and $\times 400$ (C and D, respectively) and negative control ($\times 200$) incubated with preimmune serum (A). Staining of normal lung tissue for IL-10 demonstrates the absence of detectable antigenic IL-10 at this level of sensitivity (B).

confirmed that tumor cells rather than immune cells were the primary source of antigenic IL-10 (data not shown).³⁰

Furthermore, to definitively demonstrate the capacity of bronchogenic tumors to elaborate and secrete constitutive IL-10 we used *in vitro* studies with several available bronchogenic cell lines under unstimulated conditions. The A549, A427, and Calu-6 ATCC bronchogenic tumor cell lines were cultured unstimulated for 24 hours to generate conditioned medium. The supernatants were then harvested and assessed for IL-10 by specific ELISA. As shown in Table 1, analysis of cell-free conditioned medium from these cells maintained under unstimulated conditions demonstrated 6.27 ± 1.08 , 1.87 ± 0.97 , and 7.56 ± 1.07 ng/ml antigenic IL-10, respectively, for A549, A427, and Calu-6 cell lines.

Table 1. Constitutive IL-10 Production from Bronchogenic Tumor Cell Lines

Cell Line	IL-10 (ng/ml)
A549 (CCL 185)	6.27 ± 1.08
A427 (ATCC HTB 53)	1.87 ± 0.97
Calu-6 (ATCC HTB 56)	7.56 ± 1.07

Finally, to demonstrate the functional significance of tumor-derived IL-10, we investigated the effect of conditioned medium from a bronchogenic tumor cell line on the cytokine expression of mononuclear cells. We used the A549 cell line under unstimulated conditions to generate conditioned medium. Isolated human mononuclear cells were cultured in this conditioned medium also under unstimulated conditions in the presence of control antibodies or specific neutralizing antibodies to IL-10.

As shown in Table 2, the addition of anti-IL-10 neutralizing antibodies to the conditioned medium of the A549 cell line at the time of exposure to mononuclear

Table 2. Cytokine Production from Mononuclear Cells Cultured in the Presence of Conditioned Medium from A549 Tumor Cells

	TNF (ng/ml)	IL-6 (ng/ml)
Mononuclear cells alone	0.41 ± 0.04	2.11 ± 0.37
Mononuclear cells + control Ab	0.19 ± 0.21	1.87 ± 0.13
Mononuclear cells + anti-IL-10	1.74 ± 0.26	3.49 ± 0.63

cells resulted in increased monocyte TNF- α production compared with the addition of control antibodies. The resulting TNF- α levels in mononuclear cell supernatants were 1.74 ± 0.26 and 0.19 ± 0.21 ng/ml, respectively, for cells cultured with anti-IL-10 and control antibodies ($P < 0.05$). Similar, although not statistically significant, results were obtained when supernatants were evaluated for IL-6 production. Mononuclear cells cultured with control antibodies produced 1.87 ± 0.13 ng/ml IL-6, whereas cells cultured with anti-IL-10 generated 3.49 ± 0.63 ng/ml IL-6. The addition of control antibodies to mononuclear cell cultures resulted in no significant differences in IL-6 or TNF- α production.

Discussion

The clinical response to the treatment of human bronchogenic carcinoma is dismal. Despite extensive research and numerous clinical trials using various multimodality therapies, the 5-year survival rate remains less than 15%.³¹ Although results to date have been mostly discouraging, most immunologists and cancer biologists feel that immunotherapy modalities represent the greatest promise for successful therapy for bronchogenic carcinoma and many other malignancies. A greater understanding of the interactions between tumors and the immune system is essential for the development of successful approaches with immunotherapy. Once the precise mechanisms used by tumors to evade host responses are understood, specific strategies can be used to circumvent these mechanisms and maximize immune effector cell activity.

Our study has noted significant increases in antigenic IL-10 detected in homogenates of human non-small cell bronchogenic carcinomas compared with normal lung tissue. This disparity was consistent for tumors of squamous cell and adenocarcinoma histologies. Further studies using immunohistochemical staining techniques graphically demonstrated individual tumor cells to be the primary source of antigenic IL-10. Investigations identifying mononuclear cells infiltrating tumor tissue revealed that local IL-10 was primarily tumor derived rather than a product of immune cells. The detection of significant quantities of IL-10 in the supernatants of several bronchogenic carcinoma cell lines definitively demonstrates the capacity of bronchogenic carcinomas to elaborate IL-10. Importantly, viability studies of these cell lines suggests that IL-10 may be actively secreted by these cells. Finally, functional studies examining the effects of conditioned medium from a bronchogenic carci-

noma cell line on the capacity of mononuclear cells to elaborate cytokines revealed a specific inhibitory action by tumor-derived IL-10, which importantly was attenuated by the addition of anti-IL-10.

These results demonstrate that biologically active IL-10 is constitutively produced and is probably secreted by human bronchogenic carcinomas. Elevations of IL-10 within tumors may dramatically impair local host immune responses at a number of locations along immune pathways. Sentinel macrophages infiltrating tumor tissue would encounter increasing levels of IL-10. Sufficient levels of IL-10 may then exert inhibitory actions on these mononuclear cells, resulting in a marked diminution of their cytokine producing and antigen presentation capacities. Impaired macrophage function also indirectly inhibits T cell responses due to ineffective cell-to-cell communications. Finally, tumor-derived IL-10 may also directly affect T cell and natural killer cell activity. Thus, at nearly every level of immune defense to malignancy, IL-10 may represent a potent threat to impair host responses.

Our findings support the concept that human bronchogenic carcinomas may produce IL-10 as a means of escaping host defenses. Other workers have observed IL-10 in certain lymphoproliferative and dermal malignancies.³²⁻³⁴ Additional studies are needed to definitively establish the functional significance of tumor-derived IL-10. Subsequent investigations regarding the expression of tumor-derived IL-10 may reveal potential regulatory networks leading to the production and regulation of IL-10. These networks may exploit local tissue responses and cytokine production by both resident stromal cells and infiltrating immune effector cells. As the complexities of tumor and immune cell interaction are further elucidated potential new sites for therapeutic intervention and immunomodulation may be discovered. Ultimately, it is hoped that these insights will lead to the development of effective therapies to counter tumor defenses and impact the significant mortality due to bronchogenic carcinomas and other malignant diseases.

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